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UNIVERSITY... OF ALBERTA

DEGREE FOR WHICH THESIS WAS PRESENTED.... PH. D.

YEAR THIS DEGREE GRANTED..... 1976

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THE UNIVERSITY OF ALBERTA

HYPOTHALAMIC CONTROL OF ADRENOCORTICOTROPHIC HORMONE SECRETION IN THE GOLDFISH, *CARASSIUS AURATUS*

by

JAMES NOLAN FRYER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

FALL, 1976

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Hypothalamic control of adrenocorticotrophic hormone secretion in the goldfish, *Carassius auratus*," submitted by James Nolan Fryer in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The intraperitoneal injection of lyophilized acid extracts of the hypothalamus or telencephalon of the longnose sucker, or goldfish, significantly increased the serum corticosteroid concentration of goldfish in which endogenous ACTH secretion was suppressed by prior administration of the synthetic corticosteriod, betamethasone. Extracts prepared from sucker or goldfish cerebellum were ineffective in elevating the corticosteroid levels of betamethasone-blocked goldfish. This provides direct evidence for the presence of a corticotrophin-releasing factor (CRF) in the sucker and goldfish hypothalamus and telencephalon.

Radio-frequency lesions which destroyed both the nucleus lateral tuberis pars anterior (NLTa) and rostral pars posterior (NLTp) significantly suppressed the increase in serum corticosteroid levels (stress response) of goldfish subjected to stress. Lesions placed in the posterior hypothalamus, dorsal telencephalon, dorsal to the NLT, or small lesions in the NLTa or NLTp had no effect on the stress response of goldfish. These results suggest that a large area of the NLT is involved in the control of ACTH secretion in the goldfish. This area may be the source of the hypothalamic CRF. Lesions of the nucleus preopticus (NPO) significantly diminished the stress response of goldfish. This finding suggests a role of the neurohypophyseal peptides in the control of ACTH secretion in the goldfish and may be the explanation for the presence of CRF activity in the telencephalon extracts as the NPO was included in the tissue extracted. Lesions of the epithalamus destroying the habenular nuclei resulted in an enhanced response to stress. This finding is

interpreted to indicate that neural pathways that in some way inhibit ACTH secretion are located in this brain region.

The implantation of pellets containing 0.3 µg cortisol into the third ventricle adjacent to the NLT or into the preoptic recess of the third ventricle adjacent to the NPO significantly suppressed the stress response of goldfish. Pellet implants containing 0.5 µg cortisol in the third ventricle adjacent to the NLT, the preoptic recess of the third ventricle adjacent to the NPO, or the lateral telencephalon posterior to the anterior commissure also suppressed the stress response of goldfish. However, pellets containing 0.5 µg cortisol implanted into the pituitary gland, optic tectum, or lateral telencephalon rostral to the anterior commissure, or into the third ventricle in the posterior or dorsomedial hypothalamus, had no effect on the stress response. These results provide evidence that the NLT and preoptic-telencephalon region are negative feedback sites of corticosteroids to suppress ACTH secretion in the goldfish.

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. R. E. Peter for the supervision of my graduate studies and research at the University of Alberta. I particularly thank him for his non-wavering encouragement, financial support and for the complete freedom with which he enabled me to pursue this study. His original suggestion of the research topic and critical evaluation of both the experimental work and the thesis itself are greatly appreciated.

I would like to thank my advisory committee members, Drs. D. D. Beatty, W. C. Mackay, and L. Wang, for their criticisms of this work and particularly for their review of the manuscript.

I am especially grateful to my wife, Carol, for her unrelenting moral support, for her assistance in the preparation of the graphs and diagrams of the thesis, and for typing preliminary drafts of the manuscript.

I would like to acknowledge the financial support provided by the University of Alberta in the form of two Graduate Teaching Assistantships, a Graduate Research Fellowship, and a Dissertation Fellowship.

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INTRODUCTION

The results of investigations conducted during the past decade have indicated that in teleosts the hypothalamus plays an intimate role in the control of the secretory activity of the pituitary gland. Recent evidence suggests that hormones elaborated from the teleostean adenohypophysis may be influenced by "releasing" or "inhibitory factors" of hypothalamic origin (Ball *et al.*, 1972; Peter, 1973).

It is generally accepted that in teleosts, adrenocorticotrophic hormone (ACTH) secretion may be stimulated by the hypothalamus. Evidence for such a stimulatory role comes from *in vivo* studies involving pituitary transplants. After pituitary autotransplantation in the European eel, *Anguilla anguilla* (Olivereau, 1970, 1971; Olivereau and Dimovska, 1969), and the molly, *Poecilia formosa* (Ball *et al.*, 1965), the appearance of the ACTH cells suggested reduced activity. Similarly, in the three-spined stickleback, *Gasterosteus aculeatus* (Leatherland, 1970a, b), bearing pituitary homotransplants, the ACTH cells exhibited a regressed appearance.

As an indicator of the ability of the transplanted pituitary to secrete ACTH, several investigators have examined the histological appearance of the interrenal gland. Following pituitary autotransplantation, the interrenal gland has been shown to vary from a normal appearance in *Gambusia* sp. (Chambolle, 1969, 1973) and *A. anguilla* (Olivereau, 1970, 1971; Olivereau and Dimovska, 1969), to a regressed appearance in the goldfish, *Carassius auratus* (Johansen, 1967), and the

mollies *P. formosa* (Ball *et al.*, 1965; Olivereau and Ball, 1963) and *P. latipinna* (Hawkins and Ball, 1970). However, histological criteria may not accurately reflect interrenal activity. It has been found in *P. latipinna* bearing pituitary autotransplants, that although the interrenal appeared only slightly regressed, plasma cortisol levels were at hypophysectomy levels (Hawkins and Ball, 1970). The hypofunction of the pituitary-interrenal axis generally observed following the disruption of the close anatomical relationship between the hypothalamus and the pituitary suggests a stimulatory role of the hypothalamus in ACTH secretion and the presence of a corticotrophin-releasing factor (CRF).

Additional evidence in support of a teleost CRF, comes from the investigations of Hawkins and Ball (1970) with *P. latipinna*. Following pituitary autotransplantation they observed that, although plasma cortisol concentrations were at hypophysectomy levels, the fish were capable of responding to the stress of sham-injection with increases in plasma corticoids. These authors attributed their observations to the release of a CNS neurohormone which stimulated ACTH release after transport to the pituitary via the peripheral circulation.

Subsequently, Hawkins and Ball (1973) reported on attempts to extract a CRF from teleost brain tissue. They employed dexamethasone- and betamethasone-blocked mollies, *P. latipinna*, to assay ACTH releasing agents. Dexamethasone and betamethasone are synthetic corticosteroids which block endogenous ACTH release. Injections of crude saline homogenates of the hypothalamus or the telencephalon (incorrectly referred to as olfactory lobes by Hawkins and Ball) from *P. latipinna* were ineffective in increasing plasma cortisol levels. Similarly, they found

that goldfish hypothalamic and cerebellar homogenates had no effect on plasma cortisol when injected into *P. latipinna* bearing pituitary auto-transplants. However, both acid extracts of rat median eminence tissue and synthetic arginine vasopressin were potent activators of the pituitary-interrenal axis in both intact fish, and fish bearing transplanted pituitaries.

The failure of brain tissue homogenates to stimulate ACTH secretion in *P. latipinna* (Hawkins and Ball, 1973) conflicts with the *in vitro* experiments of Sage and Purrott (1969) with cultured pituitary glands. They reported an increase in the spontaneous release of ACTH from cultured goldfish pituitaries when a goldfish hypothalamic extract was added to the incubation medium. These results provide direct evidence for the presence of a CRF in teleosts. However, the nature of such a factor or factors remains unknown.

An area of intensive research into the control of ACTH secretion in mammals has been stimulated by the finding that corticosteroids exert negative feedback effects to suppress ACTH release. Corticosteroids may inhibit ACTH release directly, by acting on the corticotrophs of the pituitary to inhibit the synthesis and/or release of ACTH, or indirectly, by acting on the brain to inhibit the synthesis and/or release of CRF. Research in this area has generated much controversy as evidence has been provided both in favour and against a pituitary feedback site and a hypothalamic feedback site for corticosteroid inhibition. The extensive literature on mammals describing this controversy has been recently reviewed by Yates and Maran (1974). These authors conclude that the existence of feedback sites for corticosteroid inhibition of ACTH

release occur at both the level of the pituitary and the brain in mammals. However, it is not known which site is the more important for corticosteroid inhibition under physiological conditions.

Metopirone (SU4885, Ciba, metyrapone) is a drug which has been shown to be a potent inactivator of 11β-hydroxylase (the enzyme responsible for the production of the 11-oxysteroids), in the mammalian adrenal cortex. Under the influence of metopirone, a decrease in the level of circulating corticosteroids occurs. Following treatment with this drug, the existence of a negative feedback system at the level of the pituitary or the hypothalamus would then result in increased activity of the ACTH cells and hypertrophy of the adrenal cortex. Administration of this drug to *A. anguilla* (Olivereau and Ball, 1963; Olivereau, 1965; Ball and Olivereau, 1966) and *P. latipinna* (Ball and Olivereau, 1966) caused hypertrophy of the interrenal cells and activation of the putative corticotrophin-secreting cells in the pituitary of each species. The metopirone-induced hypertrophy of the interrenal cells could be completely blocked in *P. latipinna*, however only partially blocked in *A. anguilla*, by the surgical removal of the pituitary gland. Metopirone has also been shown to activate the interrenal gland and the corticotrophs of *Anoptichthys jordani* (Mattheij, 1968) and *Monopterus albus* (Chan *et al.*, 1975). Thus, a negative feedback effect by the endogenous corticosteroids in teleosts seems well established. However, these studies do not differentiate between a feedback effect occurring at the level of the hypothalamus or at the level of the pituitary, since feedback action at either level will result in activation of the ACTH cells.

The ability of exogenous corticosteroids and mammalian ACTH to exert feedback effects on the ACTH cells of several teleosts has also been investigated. Cortisol injection resulted in an inactivation of the ACTH cells in *A. anguilla* (Olivereau, 1966, 1967) and *M. albus* (Chan *et al.*, 1975). In addition, partial interrenalectomy of the eel *A. anguilla* resulted in hypertrophy of the corticotrophic cells of the pituitary (Olivereau, 1964). In both *Hippocampus* (Boisseau, 1967) and *A. anguilla* (Olivereau unpublished, quoted in Olivereau, 1967) the administration of ACTH resulted in an inactivation of the ACTH cells. This would result from the exogenous ACTH stimulating the release of corticosteroids from the interrenal, which could then feed back on the pituitary or hypothalamus to suppress the activity of the ACTH cells.

The synthetic steroids, dexamethasone and betamethasone, have been used in several investigations of the pituitary-interrenal axis of teleosts. In mammals, dexamethasone and betamethasone exert feedback effects on the hypothalamus as well as the pituitary gland to suppress ACTH secretion (Smelik, 1969). Donaldson and McBride (1967) observed that the injection of dexamethasone into rainbow trout, *Salmo gairdneri*, was followed by a reduction in plasma cortisol levels. Dexamethasone has also been shown to decrease corticosteroid levels in the European eel *A. anguilla* (Bradshaw and Fontaine-Bertrand, 1968) and in the North American eel *Anguilla rostrata* (Butler *et al.*, 1969). As described earlier, Hawkins and Ball (1973) used betamethasone to block stress-induced increases in plasma corticosteroids in *P. latipinna*. Betamethasone has also been shown to be a potent blocker of the pituitary-interrenal axis of *C. auratus* subjected to a thermal stress (Fryer, 1975).

In these various experiments cited above, again the site of feedback action of betamethasone and dexamethasone in reducing circulating levels of corticosteroids in fish *in vivo* may have been at the level of the hypothalamus, the pituitary gland, or both.

Negative feedback effects of corticosteroids on the ACTH cells have been specifically demonstrated *in vitro*. Sage (1968) observed histologically an inhibition of corticotroph activity in cultured red swordtail (*Xiphophorus*) pituitaries when dexamethasone or cortisol was added to the culture medium. Similarly, with cultured goldfish pituitaries, Sage and Purrott (1969) reported a decrease in release of ACTH in the presence of cortisol.

To summarize the evidence to date, ACTH secretion in fish is stimulated by a CRF of hypothalamic origin. ACTH stimulates the release of corticosteroids from the interrenal gland and they in turn can exert negative feedback effects on the corticotrophs, or perhaps the hypothalamus, to suppress ACTH release. In addition, this feedback system may be overridden in response to various stressors which presumably act via the hypothalamus to promote further increases in ACTH secretion.

In examining the control of ACTH secretion in the goldfish, the investigations reported hereafter attempted to determine the following: if CRF activity could be demonstrated in extracts of teleost hypothalamic tissue in an *in vivo* teleost test system; the areas of the brain which may be involved in the control of ACTH secretion, and the level at which negative feedback of the adrenocorticoids occurs.

MATERIALS AND METHODS

I. EXPERIMENTAL ANIMALS AND HOLDING REGIME

Adult goldfish of both sexes (25-50 g) were obtained from a commercial supplier (Grassyfork Fisheries Co. Inc., Martinsville, Indiana) and held for a minimum of 2 weeks before experimentation in 67.5 litre aquaria (6 fish per aquarium) at a temperature of 20±1° C. Water in the aquaria was filtered through glass wool and charcoal, and changed when required. All fish were subjected to a photoperiod of 14 h light:10 h dark, and fed daily with a commercially prepared diet (Purina Trout Chow; Ralston Purina Co., St. Louis, Missouri) 1 to 2 h after the onset of the light period.

II. BLOOD SAMPLING PROCEDURES

With the exception of one experiment, all blood samples were taken from goldfish via the dorsal aorta by cutting just behind the head, and the serum was collected and stored as described in Fryer (1975, see Appendix). In the noted exception, goldfish which were sampled at weekly intervals were anaesthetized and bled from the caudal vein using a 23- or 25-gauge needle fitted to a 1.0-ml tuberculin syringe. The serum was collected and stored as described in Fryer (1975, see Appendix). All blood sampling and stress experiments were performed between 1200 and 1400 hours (4 to 6 h after the onset of the light period). Individual fish in each aquarium were identified by fin clipping.

III. ADRENOCORTICOSTEROID ASSAY PROCEDURES

As an index of ACTH secretion, circulating levels of adrenocorticosteroids were determined from serum samples with a competitive protein-binding (CPB) radioassay (Murphy, 1967). Details of the CPB radioassay procedures are also described in Fryer (1975, see Appendix I).

IV. STATISTICAL ANALYSIS

Statistical differences between groups were determined by Student's 't-test' for unpaired samples. Differences were considered to be statistically significant where the P value was less than 0.05.

V. BRAIN TISSUE EXTRACTS EXPERIMENTS

Brain tissue extracts of longnose sucker, *Catostomus catostomus*, and goldfish were tested for their ability to activate the pituitary-interrenal axis of goldfish *in vivo*. Suckers were captured on their annual spring spawning migration (early May) in the Driftpile River approximately nine kilometers from its mouth on Lesser Slave Lake, in north-central Alberta. A large knife was used to section the spinal cord immediately posterior to the head and to expose the brain in the cranial cavity. Small scissors and forceps were then used to remove the brain. In the spring of 1973, both lobes of the telencephalon, and the hypothalamus were removed from the sucker brains. However, in 1975, both lobes of the telencephalon, the hypothalamus, and a portion of the cerebellum were collected. All tissues were immediately frozen on dry ice, and later transferred to a laboratory freezer where they were

stored at -20° C.

In September 1975, goldfish held in large tanks in the Aquatic Facilities of the Department of Zoology at the University of Alberta (at 20° C on a simulated natural photoperiod) were netted, the spinal cord sectioned just behind the head with scissors, and the brain exposed. Using small scissors and forceps, both lobes of the telencephalon, the hypothalamus, and a portion of the cerebellum were removed. All tissues were immediately frozen on dry ice, and later transferred to a laboratory freezer and stored at -20° C.

Brain tissue extracts were prepared using a technique modified from Chan *et al.* (1969). Brain tissues from 200 fish of each species were homogenized at 0° C in a glass tissue homogenizer (130DX100mmL, Pyrex No. 7725) containing 25 µl of 0.1 N HCl per hypothalamus, cerebellum, or pair of telencaphalon lobes. The extract material was then boiled for 12 min and centrifuged at 7,700 g for 60 min at 2° C. The supernatant was lyophilized and the resulting powder stored at -20° C until use. Immediately prior to bioassay, the extract powders were dissolved in 1.0 ml 0.1 N NaOH and neutralized with 0.5 N NaOH. The volume of extract material was adjusted with distilled water such that in each experiment all fish received the same injection volume. Injection volumes are given in the Results section in the captions to Figures 1, 2 and 3. For the longnose suckers collected in 1975, the total wet weights of the 200 hypothalami, telencephalon lobes, and cerebellum fragments extracted were 8.03, 9.81 and 8.25 g respectively. The total wet weights of the 200 goldfish hypothalami, telencephalon lobes, and cerebellum fragments extracted were 3.55, 4.07 and 3.85 g respectively.

Extract material was injected intraperitoneally into goldfish in which endogenous ACTH secretion had been suppressed with betamethasone (9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-20-dione; Sigma). The betamethasone (0.25 mg/ml in sesame oil; injection volume 4.0 µl/g) was injected (1 mg/kg) intraperitoneally 24 h prior to the extract material. All injections were given with a 1.0-ml tuberculin syringe fitted with a 27-gauge hypodermic needle. Goldfish were bled from the dorsal aorta 45 min after receiving the extract material or an equal amount of saline (0.1 N NaCl), and serum corticosteroid levels determined as described above.

VI. HYPOTHALAMIC LESION EXPERIMENTS

Lesions were placed in the hypothalamus and other brain areas in adult goldfish employing the procedures developed and described by Peter (1970), and Peter and Gill (1975). Lesions were produced using a Grass Model LM-4 radio-frequency lesion-maker, and size 0 insect pins insulated to within 0.5 mm of the tip with Insul-X as the lesioning electrode. Voltages ranging from 40 to 80 volts were applied to the electrode for 30 sec. Sham-operated fish were prepared identically to the lesioned fish with the exception that no voltage was applied to the electrode following electrode placement. Table I summarizes the areas of the brain lesioned using coordinates for the electrode placement from the Peter and Gill (1975) atlas of forebrain nuclei in the goldfish, as well as the voltage applied in each case.

The effects of these lesions on ACTH secretion were assessed by determining the ability of lesioned fish to respond to stress with an

TABLE I

Anatomical location	Lesioning voltage (volts)	Coordinates* of electrode placement (mm)
nucleus lateral tuberis (pars anterior)	40	+0.9, midline, depth 3.2
nucleus lateral tuberis (pars posterior)	40, 60	+0.6, midline, depth 3.4
nucleus lateral tuberis (pars lateralis)	40	+0.9, right and left 0.5, depth 3.2
nucleus lateral tuberis (pars inferior)	40	+0.3, midline, depth 2.4
nucleus preopticus	40, 80	+1,0, midline, depth 2.0
nucleus habenularis	80	+0.2, midline, depth 0.6
posterior hypothalamus	80	-0.1, midline, depth 3.4
nucleus anterior tuberis	60	+0.6, midline, depth 2.9
dorsal telencephalon	80	+1.0, right and left, 0.5, depth 0.8

*Peter and Gill (1975) atlas for forebrain nuclei of the goldfish.

increase in serum corticosteroid levels. A description of the various stress protocols has been given elsewhere (Fryer, 1975; see Appendix). Briefly, for a sham-injection stress, goldfish were netted and a 27-gauge hypodermic needle was inserted 1.3 cm into the peritoneal cavity. The fish were then sampled 15 min after the return to their holding tank. A shallow-water stress consisted of netting the fish and transferring them to a bucket containing water 2.0 cm in depth where the fish were held for 15 min prior to sampling. As a thermal stress, fish were netted and transferred immediately into warm water (35° C) for a 10-min period, after which they were returned to their holding tank (water temperature 20° C) and sampled 30 min later.

Areas of the brain destroyed by the lesioning procedure were determined histologically. Brains and the attached cranial floor were dissected from the fish, fixed in Bouin's fluid, decalcified with RDO (DuPage Kinetic Laboratories, Downer's Grove, Illinois), embedded in Paraplast Plus (Sherwood Medical Industries, Downer's Grove, Illinois) and sectioned at 8 μ. The sections were stained with paraldehyde fuchsin, counterstained with acid fuchsin, ponceau xylidine and fast green (Epple, 1967).

VII. HORMONE PELLET IMPLANT EXPERIMENTS

To investigate the possibility that corticosteroids might exhibit feedback effects on the brain in controlling ACTH secretion, hormone pellets containing cortisol were implanted in various areas of the brain. Table II summarizes pellet implant sites in the brain using coordinates from the Peter and Gill (1975) atlas of forebrain nuclei in the goldfish.

To prepare material for implantation, 13.6, 22.9 or 46.9 mg crystalline cortisol (Δ^4-pregnen-11β,17a,21-triol-3,20-dione, Sigma) were added to each gram of melted cocoa butter in a small vial. Upon solidification the cortisol-cocoa butter mixture was tamped into a 27-gauge stainless steel tube containing a wire plunger. The wire plunger was lowered inside the steel tube until it was 0.3 mm from the end of the tube as judged by comparison to a template tube and plunger in which the plunger was fixed at a measured 0.3 mm from the end of the tube. The excess pellet material extruded from the tube by lowering the plunger was removed with tissue paper. Following implantation of the tube into the brain, the wire plunger was lowered to the base of the implant tube expelling the pellet. Hormone pellets formed in this manner had an average weight of 22.6±1.74 µg (mean ± SEM, N = 20) and were calculated to contain 0.29±.03, 0.54±.04 or 1.02±.07 µg cortisol (mean ± SEM, N = 20) on the basis of the average pellet weight and the known proportions of the mixture of cortisol to cocoa butter. Sham-operated goldfish were prepared in an identical manner with the exception that no cortisol was added to the cocoa butter. These are referred to as "blank" pellet implants.

The effects of the cortisol pellet implants on ACTH secretion were assessed by determining the ability of goldfish to respond to the sham-injection stress with an increase in serum cortisol levels, 48 h after the implantation of the hormone pellet. The hormone pellet implant sites were verified histologically as described previously for the brains bearing radio-frequency lesions.

TABLE II

Anatomical location	Cortisol (µg/pellet)	Coordinates* of implant tube placement (mm)
optic tectum	0.3, 0.5, 1.0	+0.2, right 1.0, depth 0.2
nucleus lateral tuberis	0.3, 0.5, 1.0	+0.9, midline, depth 2.9
posterior hypothalamus	0.5	-0.5 midline, depth 3.2
pituitary gland	0.5	+0.9, midline, depth 4.5
preoptic region	0.3, 0.5	+0.9, midline, depth 1.5
	0.5	+1.2, midline, depth 1.3
	0.5	+1.6, midline, depth 1.6
lateral telencephalon	0.5	+1.0, left 0.6, depth 0.8
anterior telencephalon	0.5	+2.1, midline, depth 1.2
anterior lateral telencephalon	0.5	+2.1, left 0.8, depth 0.6

*Peter and Gill (1975) atlas for forebrain nuclei of the goldfish.

TABLE III

Abbreviations

AC, anterior commissure

AP, area pretectalis

CM, corpus mamillare

HC, habenular commissure

HOC, horizontal commissure

NAH, nucleus anterioris hypothalami

NAPv, nucleus anterioris periventricularis

NAT, nucleus anterior tuberis

NCH, nucleus cerebellosus hypothalami

NDLI, nucleus diffusus lobi inferioris

NDM, nucleus dorsomedialis thalami

NH, nucleus habenularis

NL, neurointermediate lobe

NLT, nucleus lateral tuberis

NLTa, nucleus lateral tuberis pars anterioris

NLTi, nucleus lateral tuberis pars inferioris

NLTl, nucleus lateral tuberis pars lateralis

NLTp, nucleus lateral tuberis pars posterioris

NPO, nucleus preopticus

NPP, nucleus preopticus periventricularis

NPPv, nucleus posterioris periventricularis

NPT, nucleus posterior tuberis

NRL, nucleus recessus lateralis

NRP, nucleus recessus posterioris

NSV, nucleus saccus vasculosus

NVM, nucleus ventromedialis thalami

OC, optic chiasma

ON, optic nerve

OT, optic tract

OTec, optic tectum

PC, posterior commissure

PPD, proximal pars distalis

PS, pituitary stalk

PT, pituitary

RL, lateral recess of the third ventricle

RPD, rostral pars distalis

SCO, subcommissural organ

SD, saccus dorsalis

T, telencephalon

VC, valvulla cerebelli

III, third ventricle

RESULTS

I. BRAIN TISSUE EXTRACTS EXPERIMENTS

Serum corticosteroid levels of betamethasone-blocked goldfish 45 min after receiving a 100 µl injection of saline (0.1 N NaCl), or a lyophilized acid extract of the hypothalamus or telencephalon of longnose suckers collected in 1973, are shown in Figure 1. The corticosteroid concentrations in the serum of goldfish receiving a dose of extract equivalent to 6.25 hypothalami, 18.75 hypothalami, or 18.75 telencephalons were significantly higher ($p < 0.05$) than in fish receiving the saline injection. Serum corticosteroid levels of goldfish receiving dosages of extract equivalent to 6.25 hypothalami, 18.75 hypothalami, or 18.75 telencephalons did not differ significantly from each other.

Serum corticosteroid levels of betamethasone-blocked goldfish 45 min after receiving a 150 µl injection of saline (0.1 N NaCl), or lyophilized acid extract of the hypothalamus, cerebellum, or telencephalon of longnose suckers collected in 1975 are shown in Figure 2. The corticosteroid concentrations in the serum of goldfish receiving a dose of extract equivalent to 6.25 hypothalami, 6.25 telencephalons, 6.25 cerebellum fragments, and 18.75 cerebellum fragments were not significantly different ($p > 0.05$) from those in goldfish receiving the saline injection. Goldfish receiving the equivalent to 18.75 hypothalami or 18.75 telencephalons had corticosteroid levels which were significantly greater ($p < 0.001$) than all other groups. In addition, goldfish receiving the equivalent of 18.75 hypothalami had corticosteroid

Figure 1. Serum corticosteroid levels (mean ± SEM) of betamethasone-blocked goldfish 45 min after receiving a 100 µl intraperitoneal injection of saline (0.1 N NaCl), or lyophilized acid extract of longnose sucker brain tissue. 18.75 Tel, fish injected with a dose equivalent of 18.75 telencephalons; 6.25 Hypo, 18.75 Hypo, fish injected with a dose equivalent of 6.25 or 18.75 hypothalami, respectively. Numbers indicate the number of fish in each test group.

a, significantly greater ($p < 0.05$) than fish receiving saline.

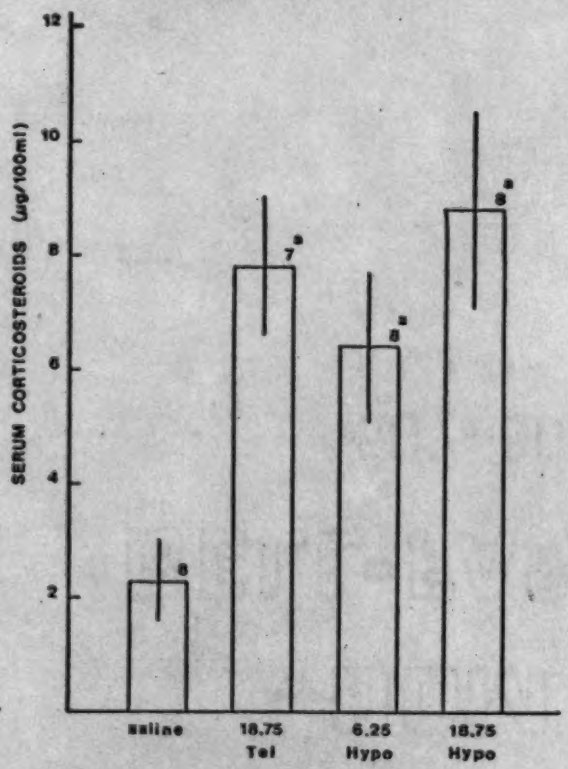

SERUM CORTICOSTEROIDS (µg/100ml)
12
10
8
6
4
2
8
7*
5*
8*
saline
18.75
Tel
6.25
Hypo
18.75
Hypo

Figure 2. Serum corticosteroid levels (mean ± SEM) of betamethasone-blocked goldfish 45 min after receiving a 150 µl intraperitoneal injection of saline (0.1 N NaCl), or lyophilized acid extract of longnose sucker brain tissue. 6.25 Hypo, 18.75 Hypo, fish injected with a dose equivalent of 6.25 or 18.75 hypothalami, respectively; 6.25 Tel, 18.75 Tel, fish injected with a dose equivalent of 6.25 or 18.75 telencephalons, respectively; 6.25 Cer, 18.75 Cer, fish injected with a dose equivalent of 6.25 or 18.75 cerebellum fragments, respectively. Numbers indicate the number of fish in each test group.

a, significantly greater ($p < 0.001$) than saline, 6.25 Hypo, 6.25 Tel, 6.25 Cer, or 18.75 Cer.

b, significantly greater ($p < 0.001$) than saline, 6.25 Hypo, 6.25 Cer or 18.75 Cer; significantly greater ($p<0.01$) than 6.25 Tel.

c, significantly greater ($p < 0.025$) than 18.75 Tel.

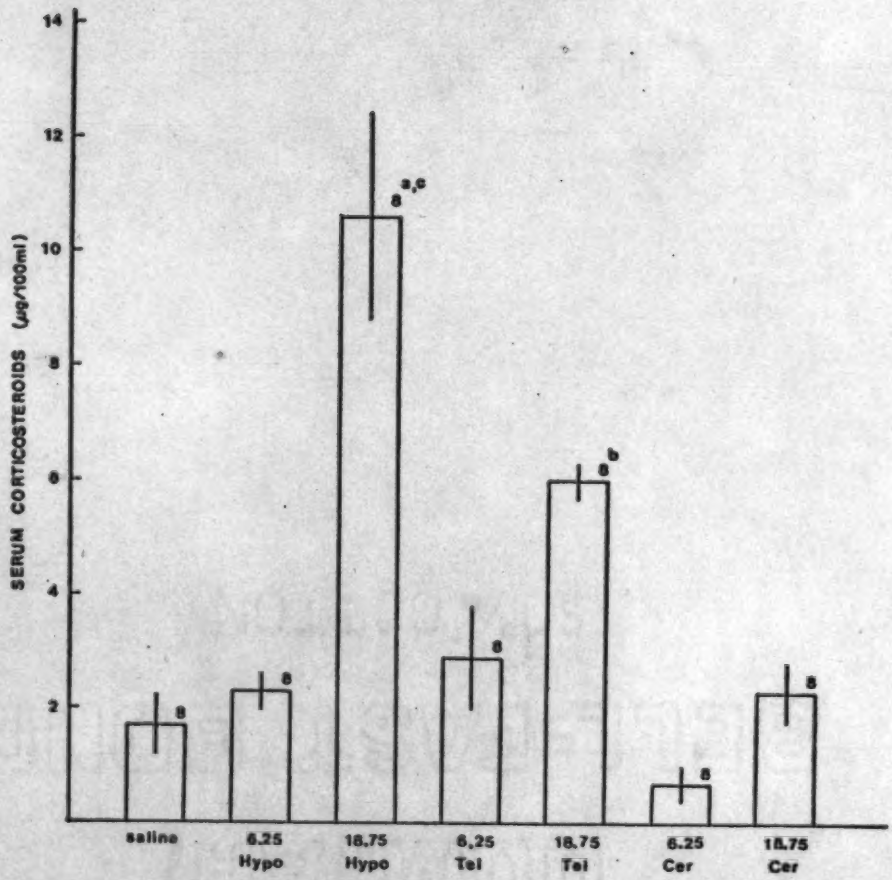
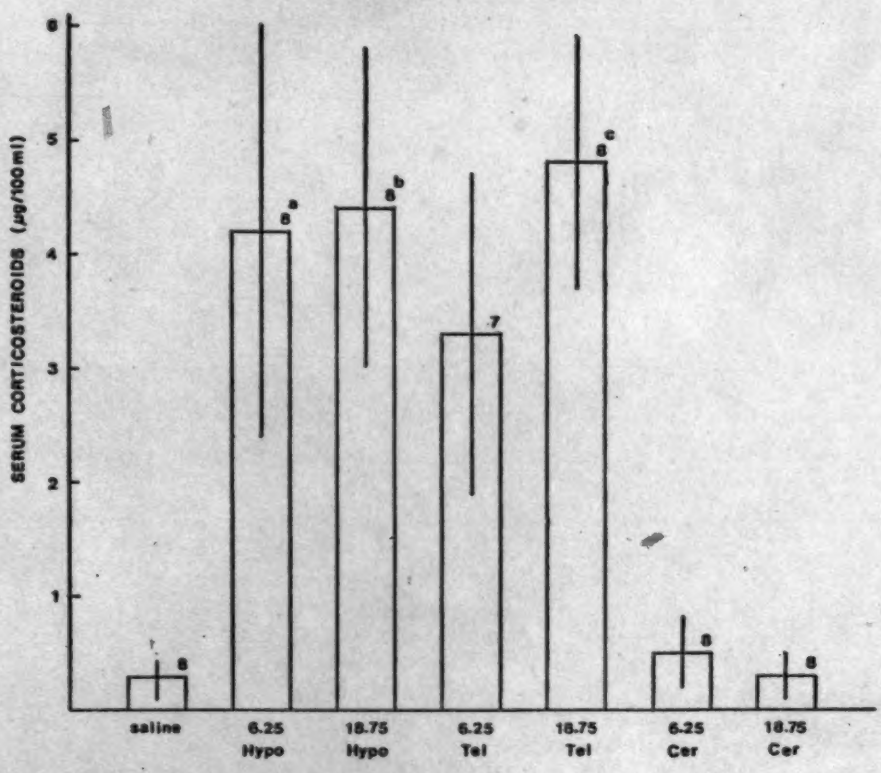

SERUM CORTICOSTEROIDS (µg/100ml)
14
12
10
8
6
4
2
8
8
8
a,c
8
8
b
8
8
8
saline
6.25 Hypo
18.75 Hypo
6.25 Tel
18.75 Tel
6.25 Cer
18.75 Cer

Figure 3. Serum corticosteroid levels (mean ± SEM) of betamethasone-blocked goldfish 45 min after receiving a 170 µl intraperitoneal injection of saline (0.1 N NaCl), or lyophilized acid extract of goldfish brain tissue. 6.25 Hypo, 18.75 Hypo, fish injected with a dose equivalent of 6.25 or 18.75 hypothalami, respectively; 6.25 Tel, 18.75 Tel, fish injected with a dose equivalent of 6.25 or 18.75 telencephalons, respectively; 6.25 Cer, 18.75 Cer, fish injected with a dose equivalent of 6.25 or 18.75 cerebellum fragments, respectively. Numbers indicate the number of fish in each test group.

a, significantly greater ($p < 0.05$) than saline, 6.25 Cer or 18.75 Cer.

b, significantly greater ($p < 0.025$) than saline, 6.25 Cer or 18.75 Cer.

c, significantly greater ($p < 0.005$) than saline, 6.25 Cer or 18.75 Cer.


SERUM CORTICOSTEROIDS (µg/100 ml)
1
2
3
4
5
6
8
8a
8b
7
8c
8
8
saline
6.25 Hypo
18.75 Hypo
6.25 Tel
18.75 Tel
6.25 Cer
18.75 Cer

titres which were significantly higher ($p < 0.025$) than fish receiving the equivalent of 18.75 telencephalons.

Serum corticosteroid levels of goldfish 45 min after receiving a 170 µl injection of saline (0.1 N NaCl), or lyophilized acid extract of the hypothalamus, cerebellum, or telencephalon of goldfish are shown in Figure 3. The corticosteroid concentrations in the serum of goldfish receiving a dose of extract equivalent to 6.25 hypothalami, 18.75 hypothalami, and 18.75 telencephalons were significantly higher ($p < 0.05$) than in goldfish receiving the saline injection or the equivalent to 6.25 cerebellum fragments, or 18.75 cerebellum fragments. Goldfish receiving a dose of extract equivalent to 6.25 telencephalons had serum corticosteroid levels which were not significantly greater ($p > 0.05$) than goldfish receiving the saline injection or the equivalent of 6.25 cerebellum fragments or 18.75 cerebellum fragments.

II. HYPOTHALAMIC LESION EXPERIMENTS

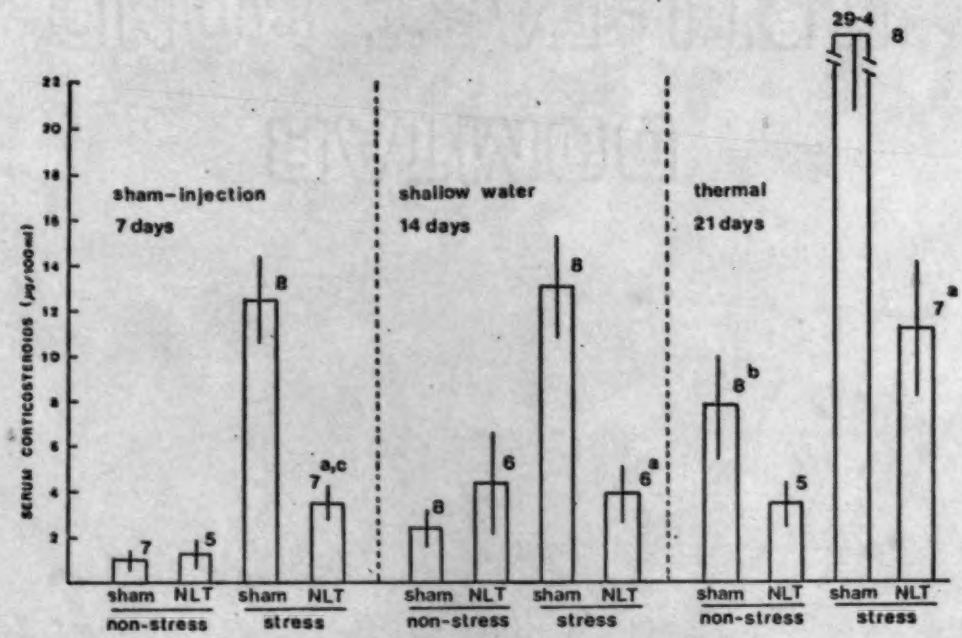
The serum corticosteroid levels of non-stressed and stressed sham-operated goldfish, or non-stressed and stressed fish bearing lesions of the nucleus lateralis tuberis (NLT) are shown in Figure 4. With non-stressed goldfish, the serum corticosteroid levels of the NLT-lesioned fish were not significantly different from the sham-operated fish at 7, 14, and 21 days post-operatively. However, the non-stress levels of corticosteroids of the sham-operated fish increased progressively with time such that the corticosteroid concentrations observed post-operatively at 21 days were significantly greater ($p < 0.05$) than those at 7 days. In response to the sham-injection, shallow water, and thermal stress

Figure 4. Serum corticosteroid levels (mean ± SEM) of non-stressed and stressed sham-operated or NLT-lesioned goldfish at 7, 14, and 21 days post-operatively. Stressed goldfish were subjected to a sham-injection stress 7 days post-operatively, a shallow water stress 14 days post-operatively, and a thermal stress 21 days post-operatively. Numbers indicate the number of fish in each group.

a, significantly lower ($p < 0.01$) than stressed sham-operated goldfish.

b, significantly greater ($p < 0.05$) than non-stressed sham-operated goldfish 7 days post-operatively.

c, significantly greater ($p < 0.05$) than non-stressed sham-operated and NLT-lesioned goldfish 7 days post-operatively.


SERUM CORTICOSTEROIDS (μg/100ml)
22
20
18
16
14
12
10
8
6
4
2
sham–injection
7 days
shallow water
14 days
thermal
21 days
29·4
8
7
5
8
7
a,c
8
6
8
6
a
8
b
5
7
a
sham
NLT
sham
NLT
sham
NLT
sham
NLT
sham
NLT
sham
NLT
non-stress
stress
non-stress
stress
non-stress
stress

protocols at 7, 14, and 21 days post-operatively, respectively, the NLT-lesioned fish had significantly lower ($p < 0.01$) serum corticosteroid levels than the sham-operated fish.

Figure 5 is a diagrammatic representation of the goldfish brain summarizing the lesioned areas for the fish "stress" group described above. Abbreviations used to describe the anatomy of the goldfish brain are summarized in Table III (p. 15).

The NLT of the goldfish is divided into various regions. The portion of the NLT continuous along the walls of the third ventricle is divided into three areas--the pars anterior, pars posterior, and pars inferior. The rostral portion of the NLT, the NLT pars anterior (NLTa), extends caudal to the posterior margin of the pituitary stalk. Figure 13 shows a cross-section through the forebrain of a normal goldfish at the level of the NLTa. The NLT pars posterior (NLTp) extends from the posterior margin of the pituitary stalk to the beginning of the lateral recess of the third ventricle. The NLT pars inferioris (NLTi) is that portion of the NLT caudal to the lateral recess of the third ventricle. The NLT pars lateralis (NLTl) appears as two nests of cells separate and lateral to the NLTa and NLTp. In the results presented in Figure 4 in which NLT lesions suppressed the stress response of goldfish, the lesioned area included the NLTa, NLTl and the rostral NLTp.

A diagram summarizing the lesioned areas for the non-stressed goldfish in this first lesion experiment is presented in Figure 6. In the results presented in Figure 4 in which NLT lesions did not affect the non-stress levels of serum corticosteroids of goldfish, the lesioned area included the NLTa and the rostral NLTp. The NLTl was completely

Figure 5. A diagram summarizing lesioned areas of the NLT of goldfish subjected to the sham-injection, shallow water and thermal stress protocols. The stippled area denotes the area of destruction common to all fish. The distance between the cross-sections is given in mm.

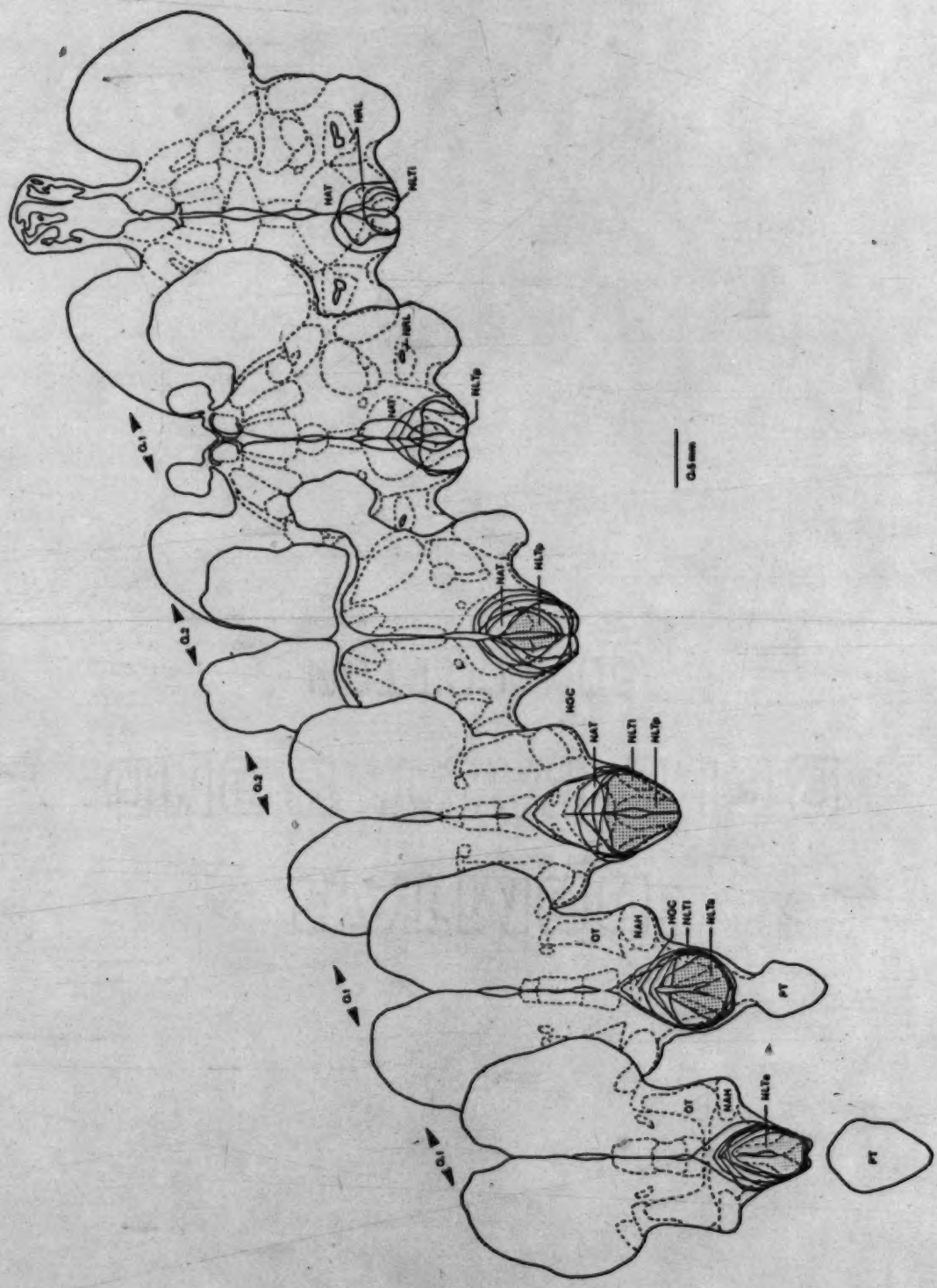
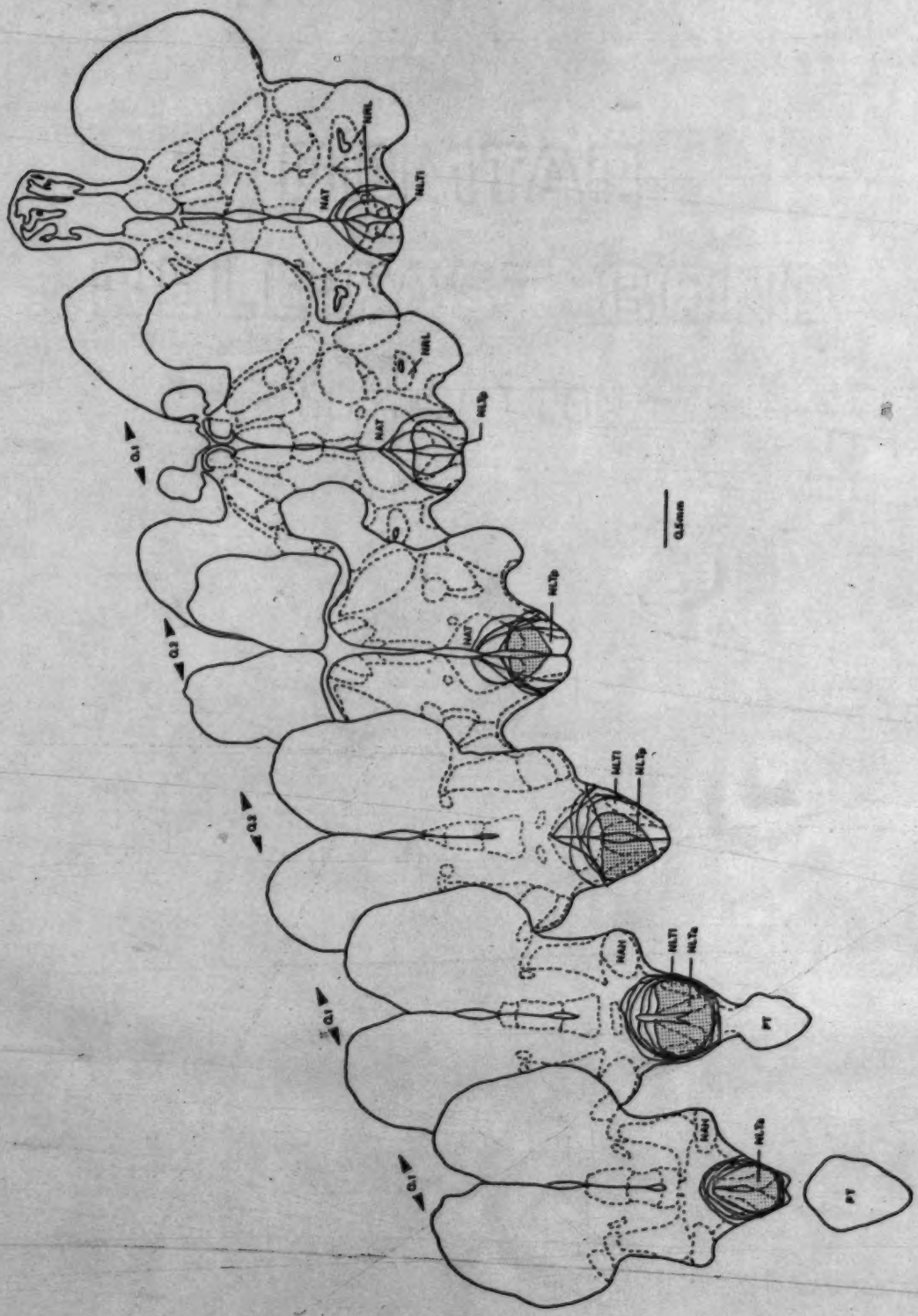

HNL
HAT
HLTi
HLTb
HAT
HLTb
HOC
HAT
HLTi
HLTb
OT
HAH
HOC
HLTi
HLTb
FT
OT
HAH
HLTb
FT
O1
O2
O3
O1
O1
0.5 mm

Figure 6. A diagram summarizing lesioned areas of the NLT of non-stressed goldfish


MAT
MLTI
MLSb
0.5mm
PT

lesioned in six fish. In a seventh fish it was incompletely lesioned with cells remaining unilaterally.

Fiber tracts of axons with cell bodies located in the nucleus preopticus (NPO) traverse the lateral regions of the ventral hypothalamus to enter the pituitary stalk and terminate in the neurointermediate lobe of the pituitary. The large lesions of the ventral hypothalamus summarized in Figure 5 and Figure 6 variously reduced the amount of stainable neurosecretory material in the neurointermediate lobe of the pituitary of the lesioned fish, indicating some damage to the fiber tracts originating in the NPO.

The effects of lesioning the NLTa or NLTp on serum corticosteroid levels of goldfish subjected to the thermal stress 21 days postoperatively are shown in Figure 7. Goldfish with lesions of the NLTa or NLTp had corticosteroid levels which were not significantly different from sham-operated and unoperated control fish. A diagram summarizing lesions of the NLTa is shown in Figure 8. The common area of destruction in these fish was restricted to that portion of the NLTa rostral to the anterior margin of the pituitary stalk. A cross-section of the brain of a goldfish with a lesion in this region of the NLTa is shown in Figure 14. A diagram summarizing the lesions of the NLTp is shown in Figure 9. A cross-section of the brain of a goldfish with a lesion in the NLTp is presented in Figure 15. Due to the small size of these lesions the common area of destruction in these fish was not extensive. The NLTp was not completely destroyed in any fish.

Figure 10 summarizes the effects of lesioning both the NLTa and rostral NLTp, or the nucleus anterior tuberis (NAT) dorsal to the NLT on the serum corticosteroid levels of goldfish subjected to a thermal stress

Figure 7. Serum corticosteroid levels (mean ± SEM) of unoperated control goldfish, sham-operated goldfish, and goldfish bearing lesions in the NLTa or NLTp following a thermal stress at 21 days post-operatively. Numbers indicate number of fish in each group.

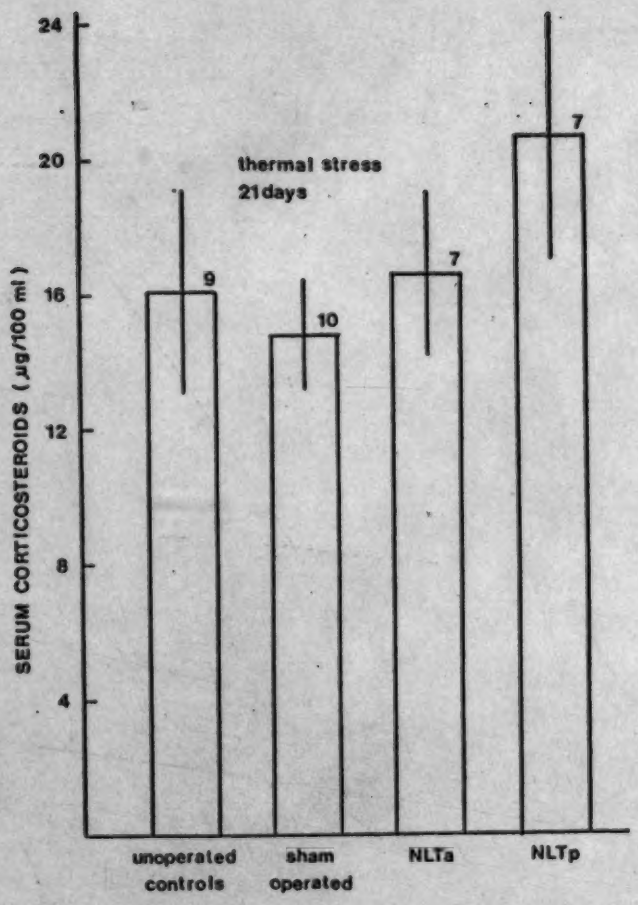

24
20
16
12
8
4
SERUM CORTICOSTEROIDS (µg/100 ml)
thermal stress
21days
9
10
7
7
unoperated controls
sham operated
NLTa
NLTp

Figure 8. A diagram summarizing lesioned areas of the NLTa of goldfish subjected to a thermal stress

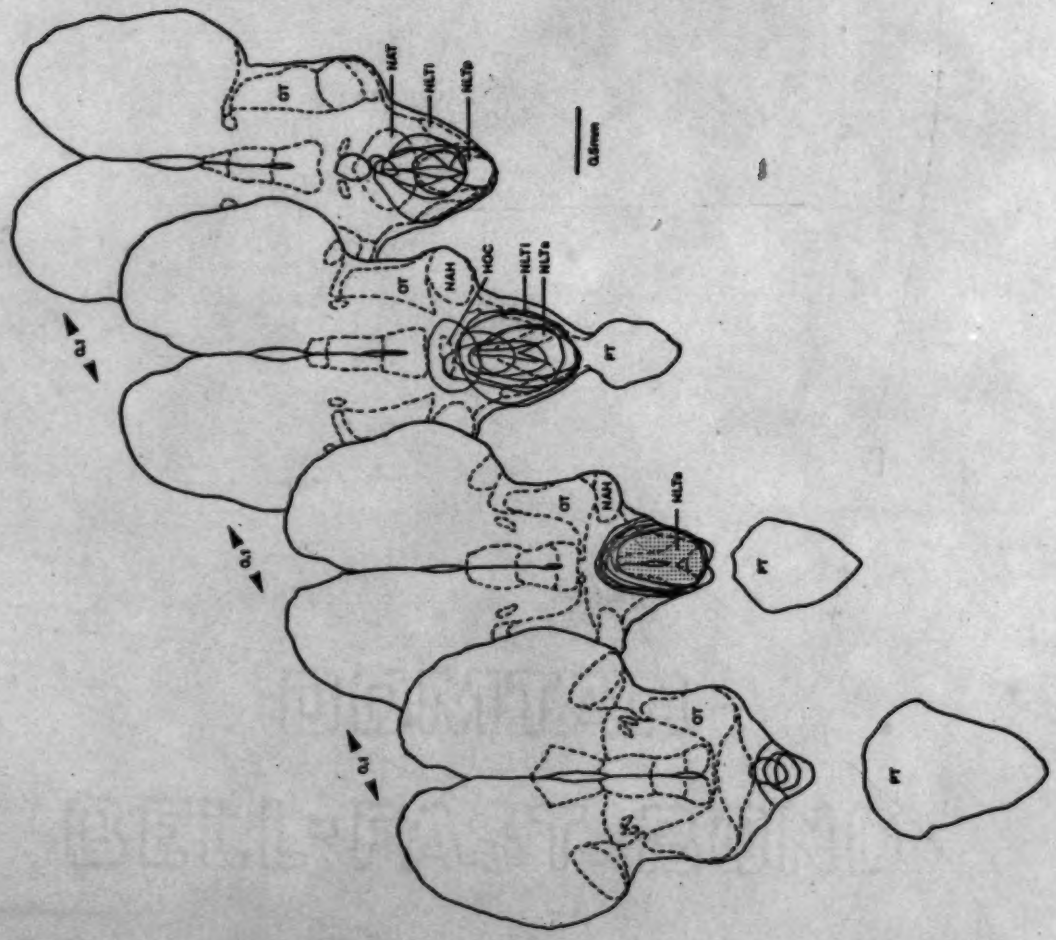

NAT
HLT1
HLTb
0.5mm
ST
NAH
HOC
HLT1
HLTb
FT
ST
NAH
HLTb
FT
ST
FT

Figure 9. A diagram summarizing lesioned areas of the NLTp of goldfish subjected to a thermal stress

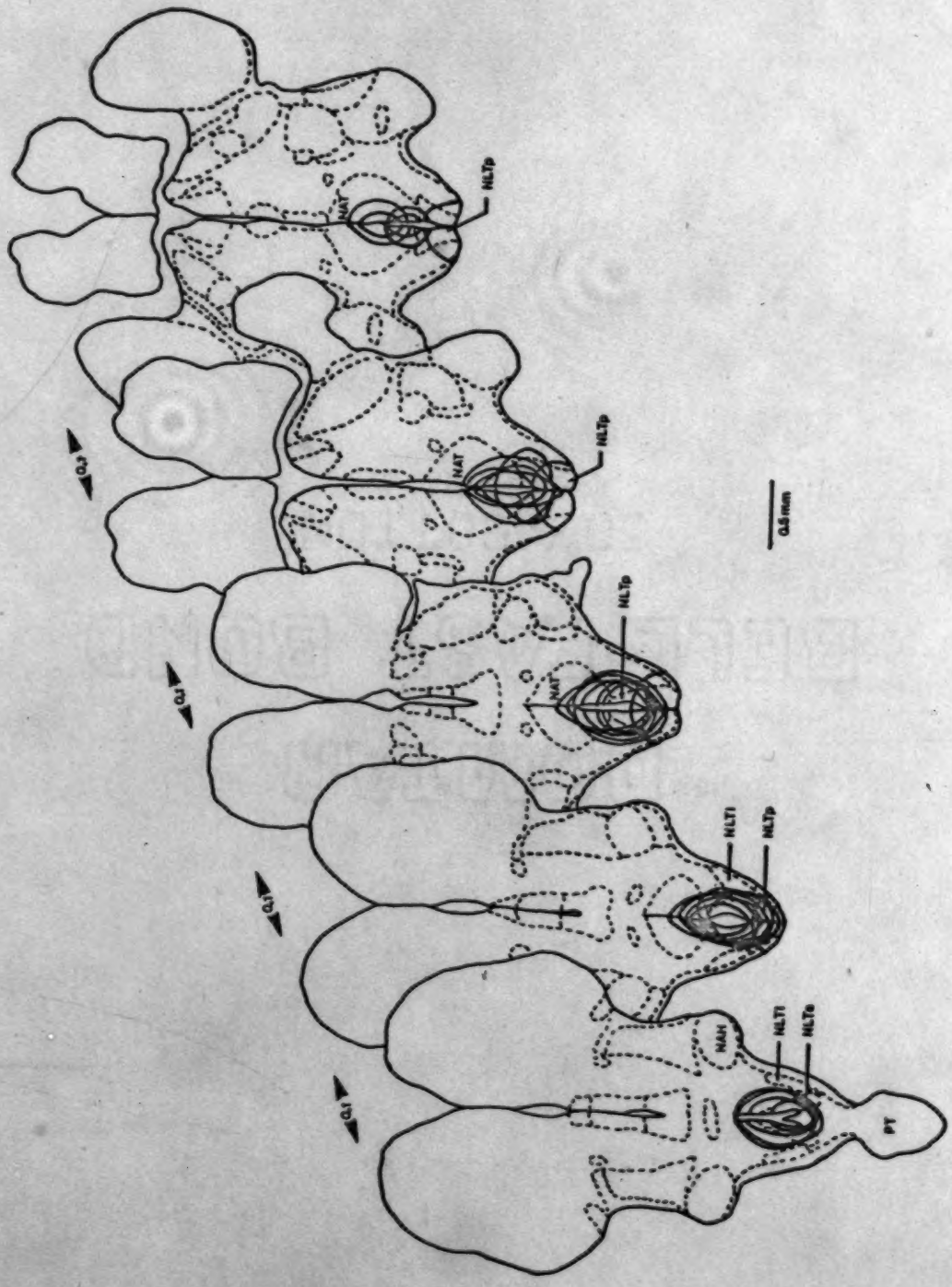

NAT
HLTp
NAT
HLTp
NAT
HLTp
NAH
HLTi
HLTe
PT
0.5 mm

Figure 10. Serum corticosteroid levels (mean ± SEM) in response to a thermal stress of sham-operated goldfish and goldfish bearing lesions of the NLTa and NLTp or NAT at 21 days post-operatively. Numbers indicate the number of fish in each group.

a, significantly lower ($p < 0.001$) than sham-operated goldfish or goldfish bearing lesions of the NAT.

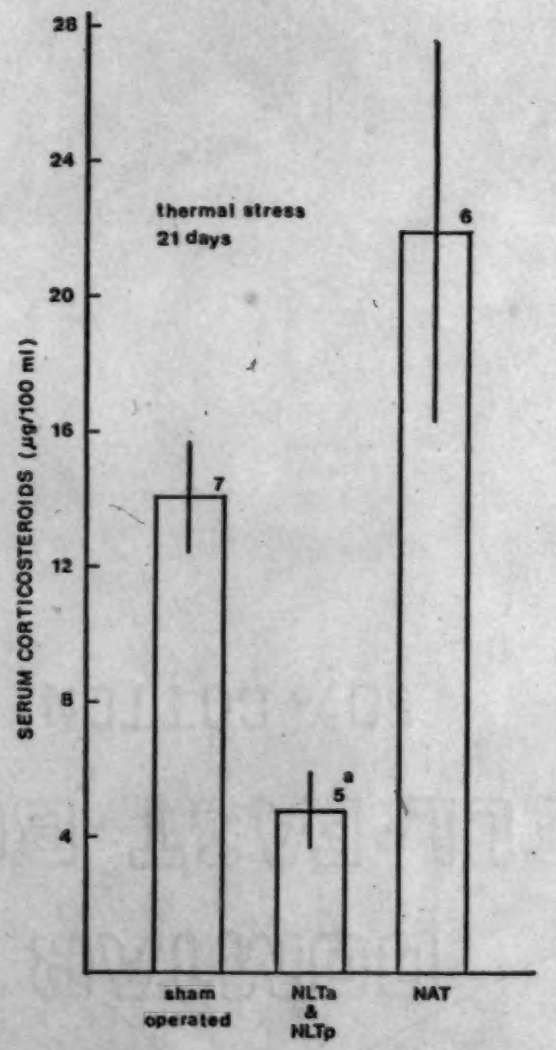

28
24
20
16
12
8
4
SERUM CORTICOSTEROIDS (µg/100 ml)
thermal stress
21 days
7
5
a
6
sham
operated
NLTa
&
NLTp
NAT

Figure 11. A diagram summarizing lesioned areas of the NLTa and NLTp of goldfish subjected to a thermal stress

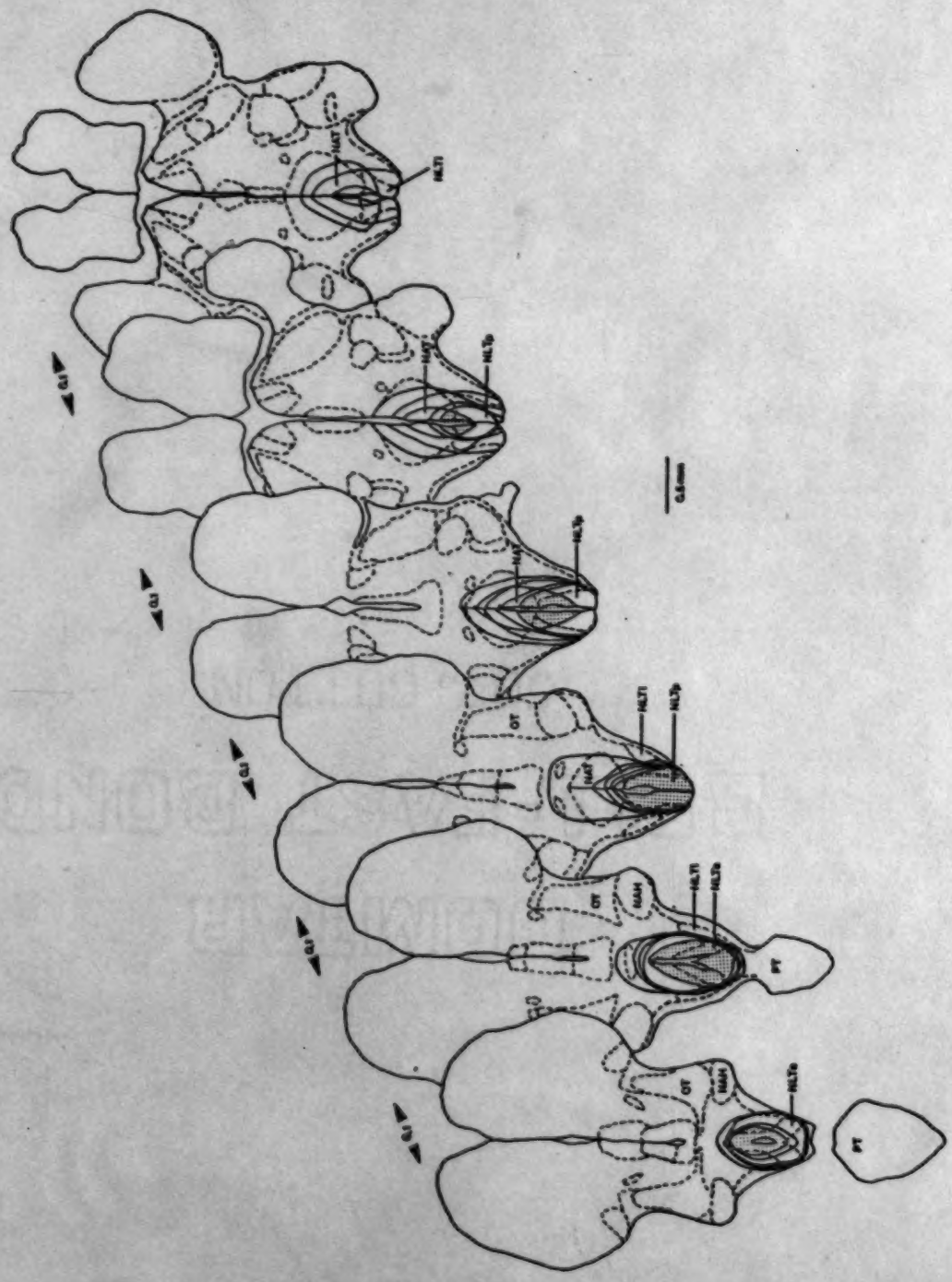

MAT
MLT
MLTb
MAH
MLTa
MLTb
MAH
MLTa
MLTb
MAH
MLTa

Figure 12. A diagram summarizing lesioned areas of the NAT of goldfish subjected to a thermal stress

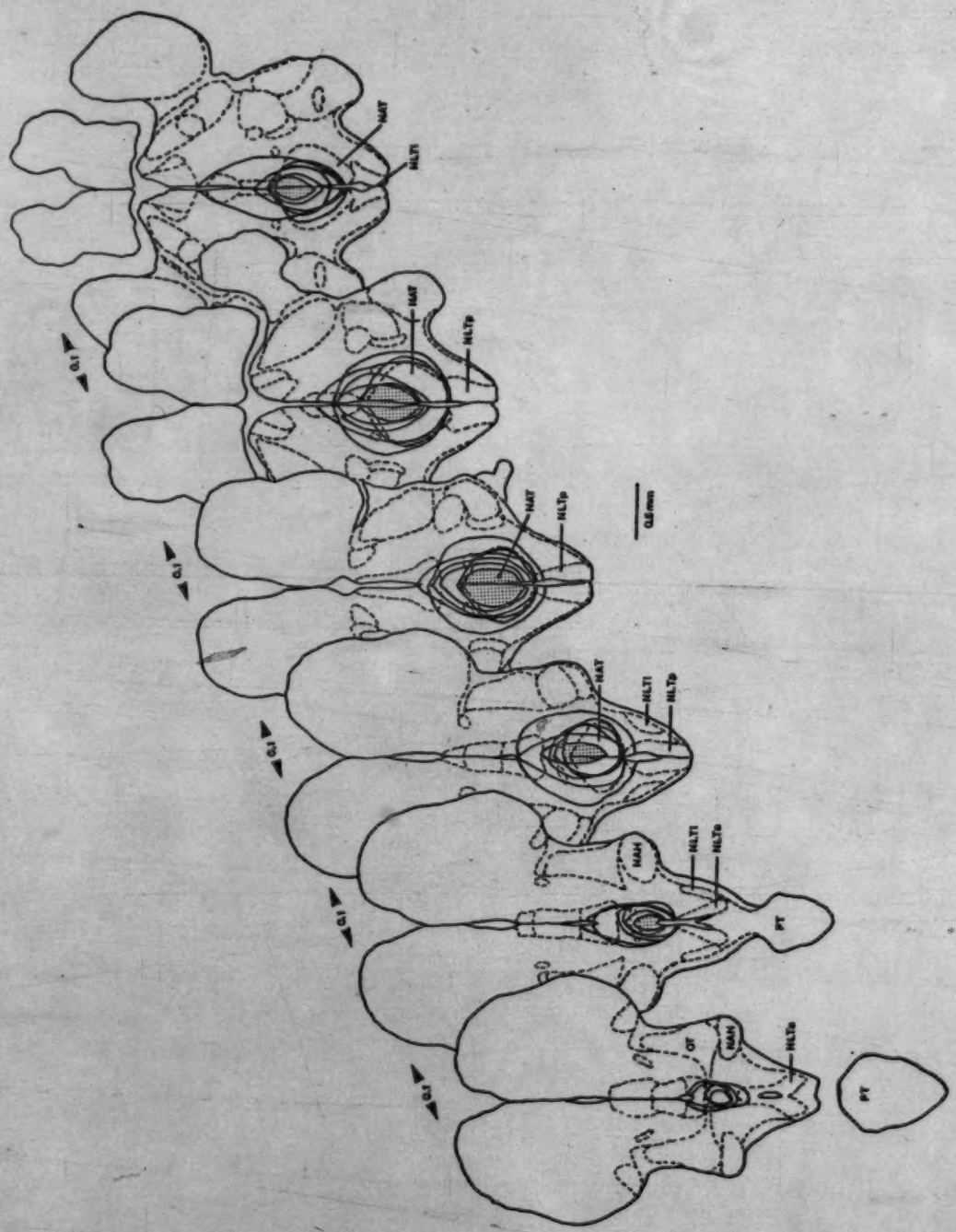
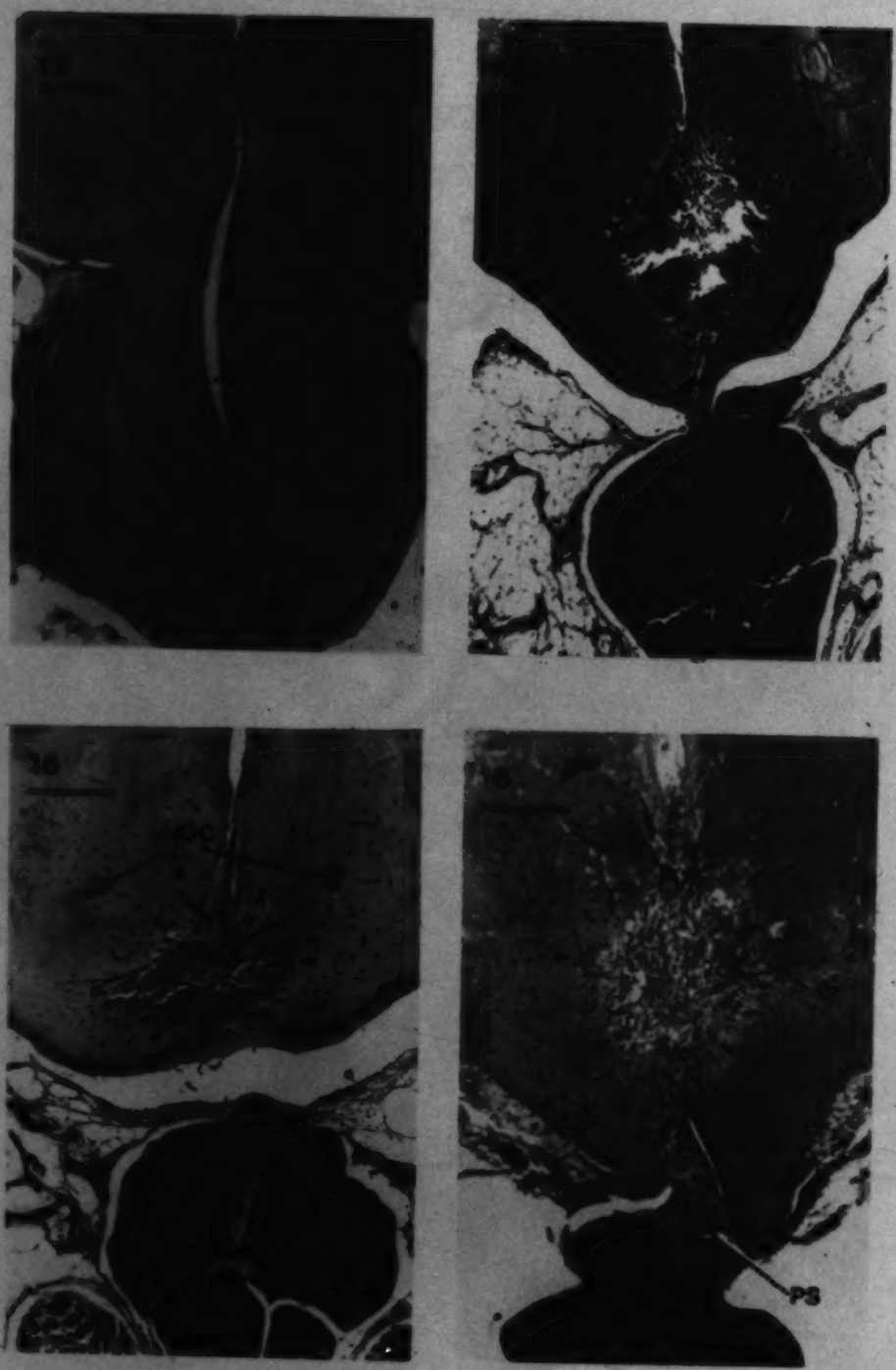

NAT
HLTI
NAT
HLTb
NAT
HLTb
NAT
HLTI
HLTb
NAH
HLTI
HLTb
PT
OT
NAH
HLTb
PT
0.5 mm

Figure 13. A cross-section through the brain of a goldfish anterior to the pituitary. Scale 200 µm.

Figure 14. A cross-section through the brain of a goldfish with a lesion of the NLTa. The lesion (arrows) has destroyed the NLTa dorsal to the pituitary stalk leaving the NLTl intact. Scale 200 µm.

Figure 15. A cross-section through the brain of a goldfish with a lesion (arrows) of the NLTp. Scale 200 µm.

Figure 16. A cross-section through the brain of a goldfish with a lesion (arrows) of the NAT. Scale 200 µm.


PS

at 21 days post-operatively. Goldfish bearing lesions of the NLTa and rostral NLTp had corticosteroid levels which were significantly lower ($p < 0.001$) than the sham-operated goldfish or the goldfish with lesions of the NAT. A diagram summarizing the lesions in the NLT is shown in Figure 11. The area lesioned included the NLTa and the rostral NLTp. The neurointermediate lobe of the pituitary of these NLT-lesioned fish had copious amounts of neurosecretory material indicating little damage to the fiber tracts from the preoptic nucleus. A diagram summarizing the lesions in the NAT is shown in Figure 12. The area lesioned included a large portion of the NAT immediately dorsal to the medial NLT. A cross-section through the brain of a goldfish with a lesion of the NAT is shown in Figure 16.

The effect of small lesions (lesioning voltage 40 volts) of the NPO on serum corticosteroid levels of goldfish subjected to a thermal stress 21 days post-operatively is summarized in Figure 17. Goldfish bearing small lesions of the NPO had corticosteroid levels which were not significantly different from sham-operated and unoperated control fish. A diagram summarizing the brain areas lesioned is presented in Figure 18. Much of the NPO in these fish remained intact both anteriorly and posteriorly. In several of these fish in which a large portion of the NPO was destroyed, the amount of stainable neurosecretory material in the neurointermediate lobe of the pituitary was markedly reduced.

The effects of large lesions (lesioning voltage 80 volts) of the NPO on the serum corticosteroid levels of goldfish subjected to a thermal stress at 21 days post-operatively are shown in Figure 19. Goldfish with large lesions of the NPO had corticosteroid levels which were significantly lower ($p < 0.001$) than the sham-operated fish. A diagram summarizing the

Figure 17. Serum corticosteroid levels (mean ± SEM) in response to a thermal stress of unoperated control goldfish, sham-operated goldfish, and goldfish bearing small lesions in the NPO at 21 days post-operatively. Numbers indicate number of fish in each group.

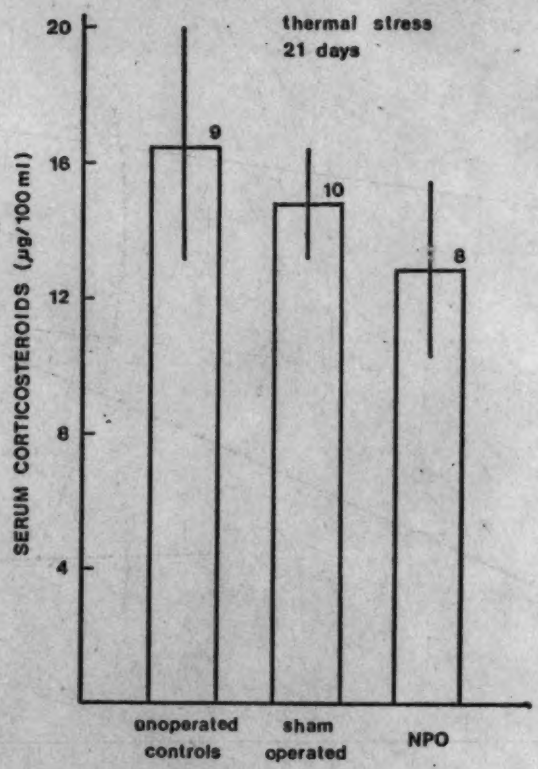
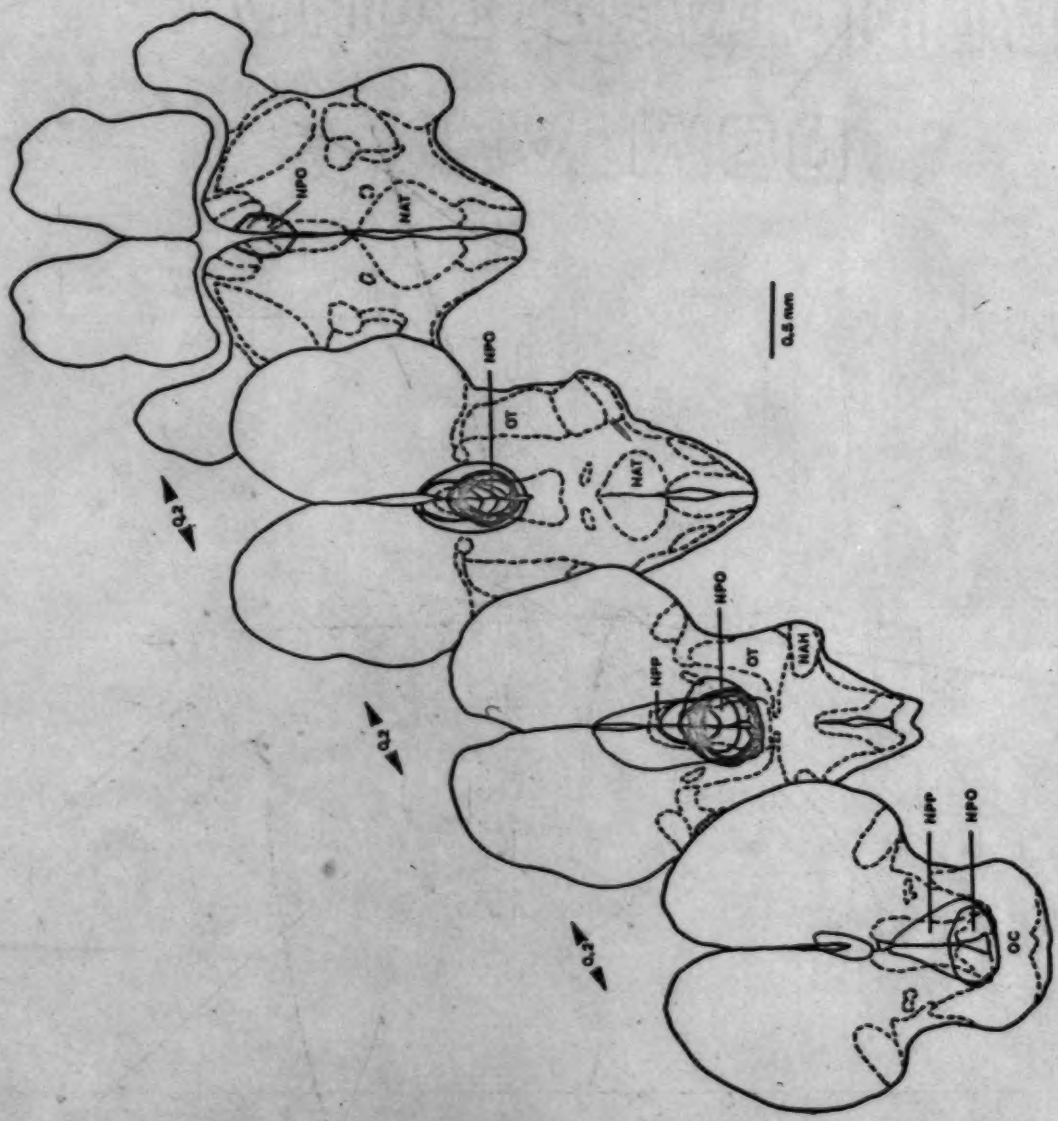

thermal stress
21 days
SERUM CORTICOSTEROIDS (µg/100 ml)
20
16
12
8
4
9
10
8
unoperated controls
sham operated
NPO

Figure 18. A diagram summarizing lesioned areas of the NPO of goldfish subjected to a thermal stress


HPO
HAT
O
0,5 mm
HPO
OT
HAT
HPP
HPO
OT
NAH
HPP
HPO
OC

lesioned areas is presented in Figure 20. These lesions destroyed a vast area of the NPO with only a small portion of the nucleus remaining intact anteriorly and posteriorly. The neurointermediate lobe of the pituitary of each fish bearing such lesions was completely devoid of neurosecretory material. A cross-section of the brain of a goldfish with a lesion of the NPO is shown in Figure 21. A section through the pituitary of a goldfish with a lesion of the NPO is presented in Figure 22. Note the absence of neurosecretory material compared to a section through the pituitary of the non-lesioned sham-operated fish shown in Figure 23.

The effect on serum corticosteroid levels of lesioning the NPO of goldfish subjected to a sham-injection stress 21 days post-operatively is summarized in Figure 24. Stressed goldfish bearing large lesions of the NPO (lesioning voltage 80 volts) had significantly lower ($p < 0.005$) corticosteroid levels than the stressed sham-operated and unoperated control fish. However, in response to the stress, the NPO-lesioned fish had corticosteroid levels which were significantly greater ($p < 0.005$) than those of non-stressed unoperated control fish. Sham-operated and unoperated control fish had similar corticosteroid levels following the stress.

A diagram summarizing the brain areas lesioned is presented in Figure 25. These lesions destroyed a large area of the NPO with only a small portion of the nucleus remaining anteriorly. The neurointermediate lobe of the pituitaries of the lesioned fish was completely devoid of neurosecretory material.

The effects of lesioning the dorsal telencephalon, posterior

Figure 19. Serum corticosteroid levels (mean ± SEM) in response to a thermal stress of sham-operated goldfish or goldfish bearing lesions of the NPO at 21 days post-operatively. Numbers indicate the number of fish in each group.

a, significantly lower ($p < 0.001$) than sham-operated goldfish.

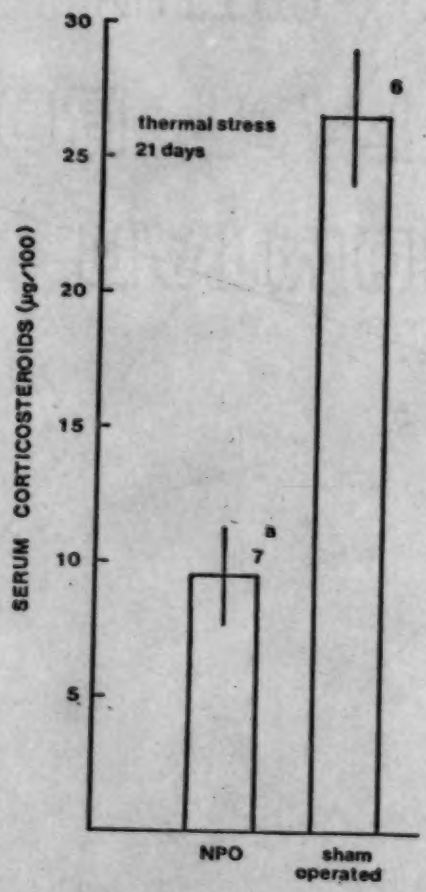

30
25
20
15
10
5
SERUM CORTICOSTEROIDS (µg/100)
thermal stress
21 days
6
7
a
NPO
sham
operated

Figure 20. A diagram summarizing lesioned areas of the NPO of goldfish subjected to a thermal stress

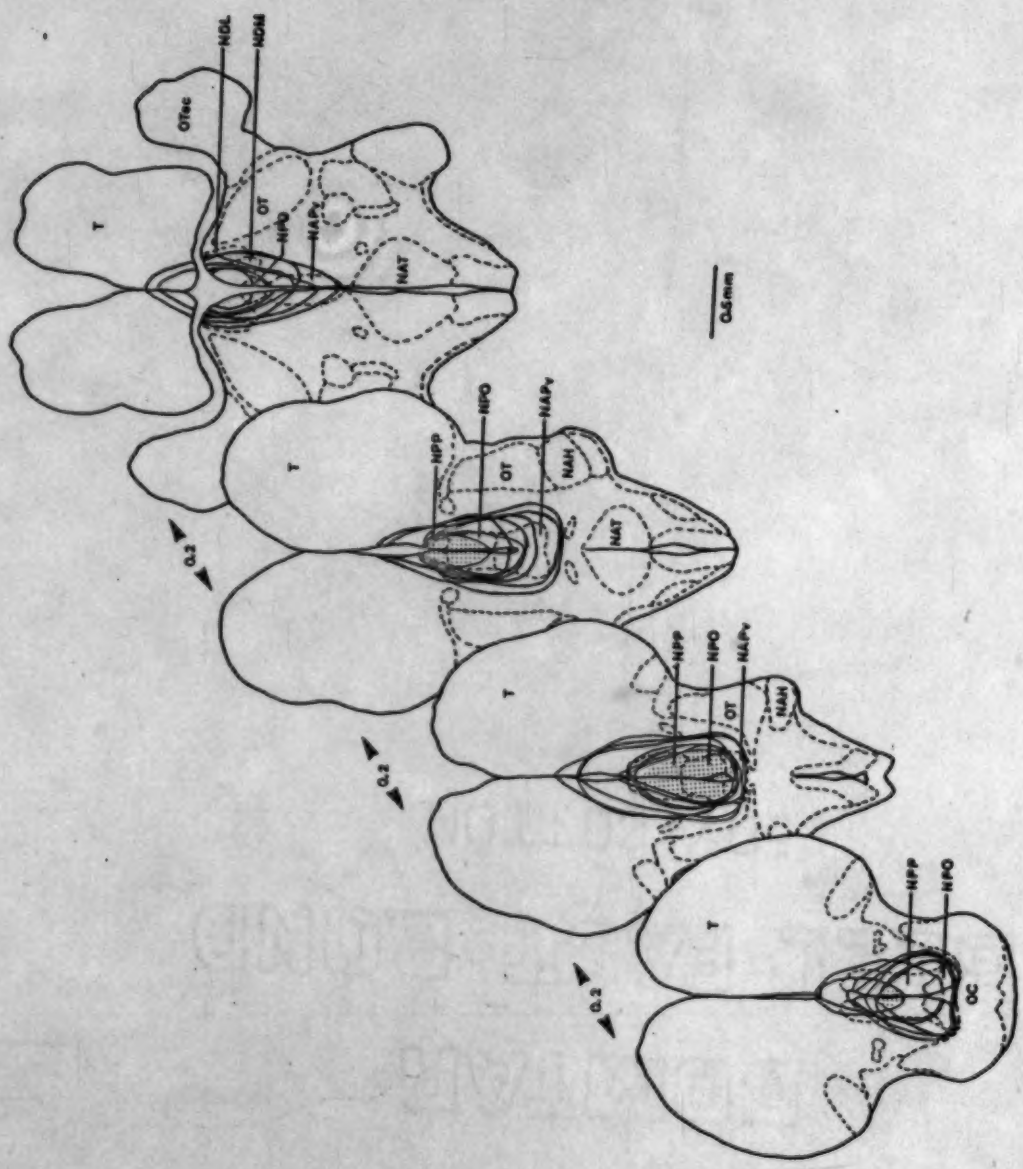

NIOL
NOM
OTac
OT
NPO
NAPv
NAT
T
0.5mm
NPP
NPO
OT
NAPv
NAH
NAT
T
0.2
NPP
NPO
OT
NAPv
NAH
T
0.2
NPP
NPO
OC
T
0.2

Figure 21. A cross-section through the brain of a goldfish with a lesion (arrows) of the NPO. Scale 200 µm.

Figure 22. A cross-section through the pituitary of a goldfish bearing a large lesion of the NPO. Note the absence of stainable neurosecretory material in the neurointermediate lobe. Scale 200 µm.

Figure 23. A cross-section through the pituitary of a sham-operated control goldfish. The neurointermediate lobe contains a large amount of stainable neurosecretion (arrows). Scale 200 µm.

21

22

Figure 24. Serum corticosteroid levels (mean ± SEM) of non-stress unoperated control goldfish, and goldfish bearing lesions of the NPO, sham-operated goldfish, and unoperated control goldfish in response to a sham-injection stress 21 days post-operatively. Numbers indicate the number of fish in each group.

a, significantly greater ($p < 0.005$) than non-stress unoperated control goldfish.

significantly lower ($p < 0.005$) than stressed sham-operated goldfish or unoperated control goldfish.

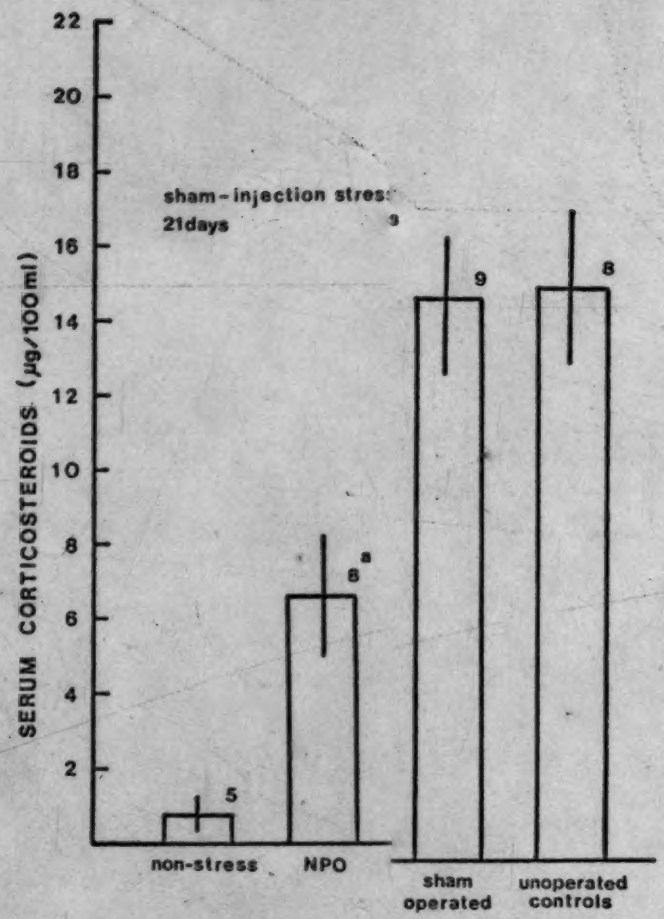
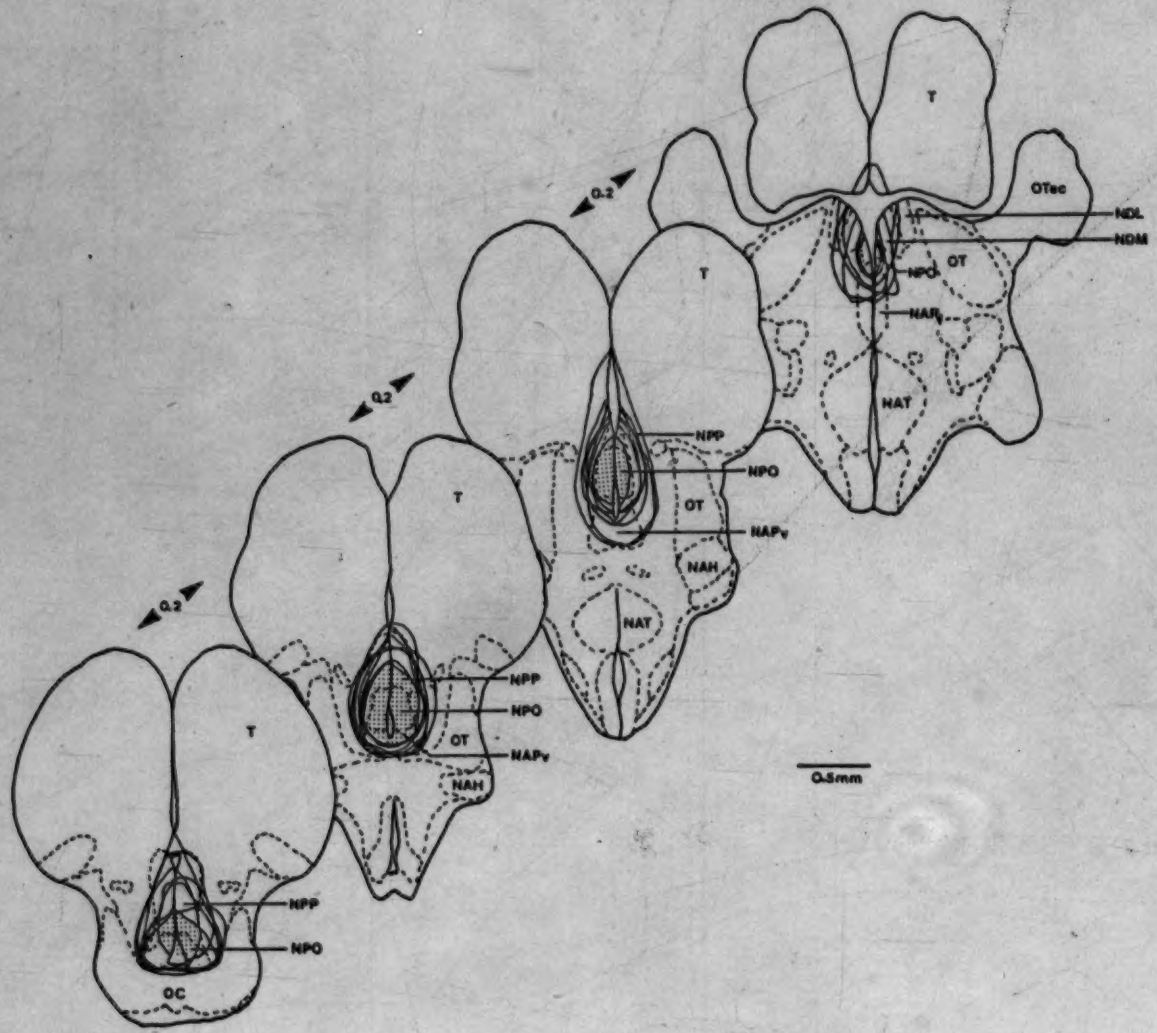

SERUM CORTICOSTEROIDS (µg/100ml)
22
20
18
16
14
12
10
8
6
4
2
sham-injection stress
21days
5
8a
9
8
non-stress
NPO
sham operated
unoperated controls

Figure 25. A diagram summarizing lesioned areas of the NPO of goldfish subjected to a sham-injection stress


T
OTec
NDL
NDM
NPd
OT
NAR
NAT
0.2
NPP
NPO
OT
NAPv
NAH
NAT
0.5mm
T
NPP
NPO
OT
NAPv
NAH
0.2
T
NPP
NPO
OC
0.2

mediobasal hypothalamus, or the habenular nuclei of the epithalamus on serum corticosteroid levels of goldfish subjected to a thermal stress at 21 days post-operatively are shown in Figure 26. Corticosteroid levels of sham-operated goldfish were not significantly different from unoperated control fish in response to the thermal stress. Goldfish with bilateral lesions in the telencephalon (Fig. 30) or lesions of the posterior mediobasal hypothalamus immediately caudal to the NLTp (Fig. 31) had corticosteroid levels which were not significantly different from unoperated control and sham-operated goldfish. However, goldfish bearing lesions in the epithalamus destroying the habenular nuclei, had corticosteroid levels which were significantly greater following a thermal stress ($p < 0.05$) than in both the unoperated control and the sham-operated fish. A cross-section through the epithalamus of an intact goldfish is shown in Figure 32. A section through the same region of the epithalamus of a goldfish in which the habenular nuclei have been completely destroyed is shown in Figure 33. The epithalamic lesions (Fig. 27) destroyed the habenular nuclei, and in 7 of 8 fish completely sectioned the posterior commissure. The telencephalon lesions (Fig. 28) were located dorsal to the NPO and the nucleus preoptic periventricularis (NPP). The area lesioned in the posterior mediobasal hypothalamus (Fig. 29) included the caudal NLTp, NLTi, nucleus saccus vasculosus (NSV), and the nucleus posterior tuberis (NPT).

III. HORMONE PELLET IMPLANT EXPERIMENTS

The serum corticosteroid levels of goldfish in response to a sham-injection stress at 48 hours after the implantation of a pellet containing

Figure 26. Serum corticosteroid levels (mean ± SEM) of unoperated control goldfish, sham-operated goldfish, and goldfish bearing lesions of the telencephalon (Tel), posterior hypothalamus (Post Hypo), and habenular nuclei (NH) in response to the thermal stress 21 days post-operatively. Numbers indicate the number of fish in each group.

a, significantly greater ($p < 0.05$) than sham-operated goldfish or unoperated control goldfish.

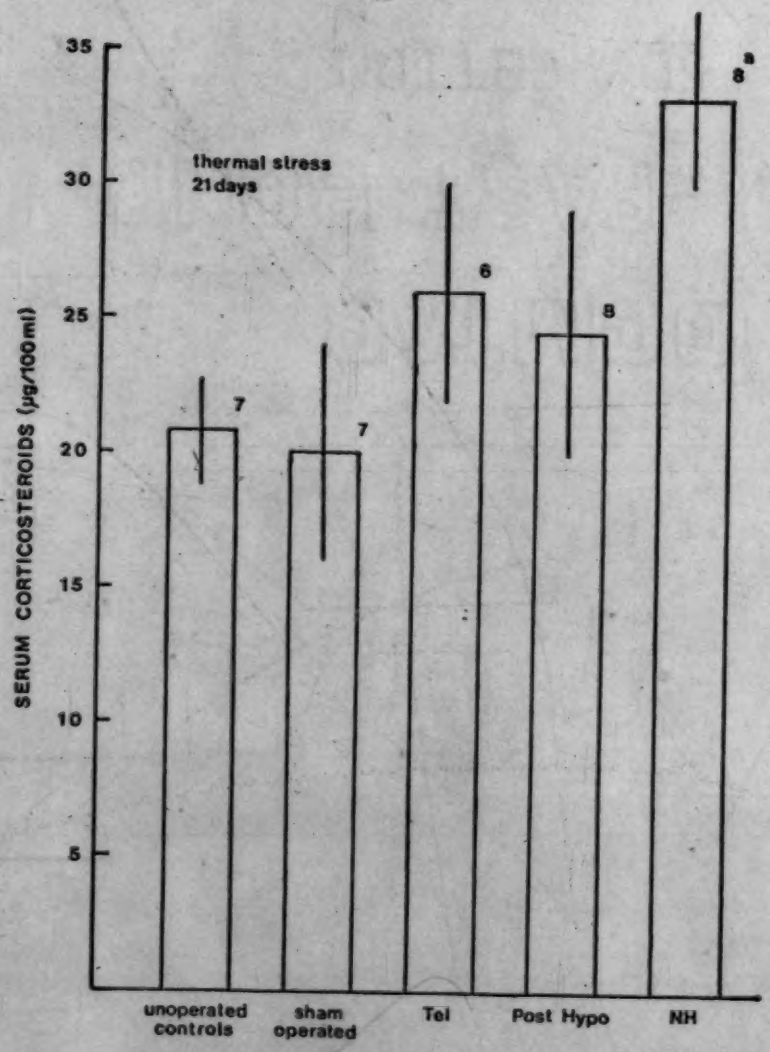

thermal stress
21 days
SERUM CORTICOSTEROIDS (µg/100ml)
35
30
25
20
15
10
5
7
7
6
8
8a
unoperated controls
sham operated
Tel
Post Hypo
NH

Figure 27. A diagram summarizing lesioned areas of the epithalamus of goldfish subjected to a thermal stress

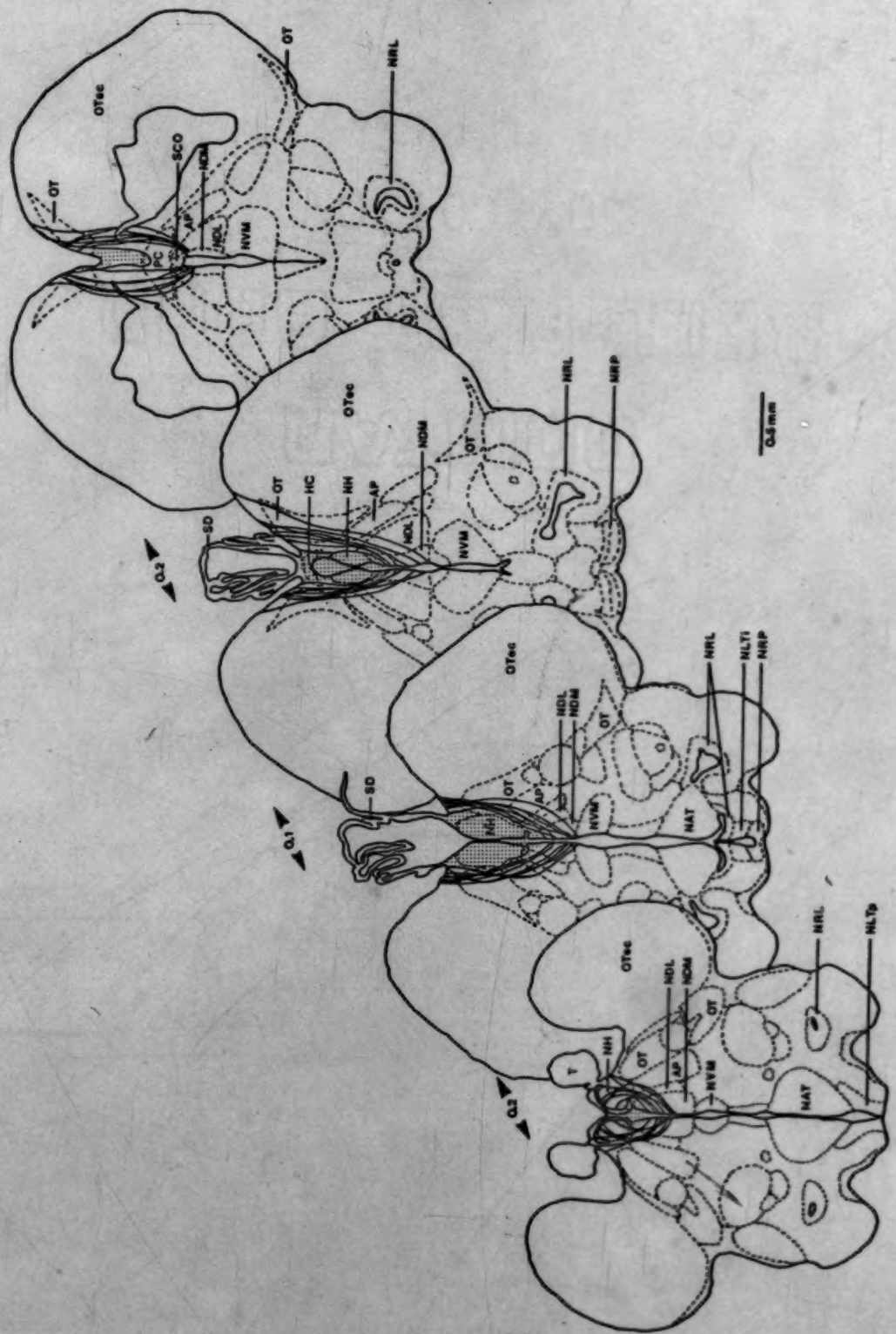

OTec
OT
NRL
SCO
NDM
AP
NDL
NVM
PC
HC
HH
NRP
SD
0.2
0.1
0.5mm
NAT
NLTi
NLTp

Figure 28. A diagram summarizing lesioned areas of the dorsal telencephalon of goldfish subjected to a thermal stress

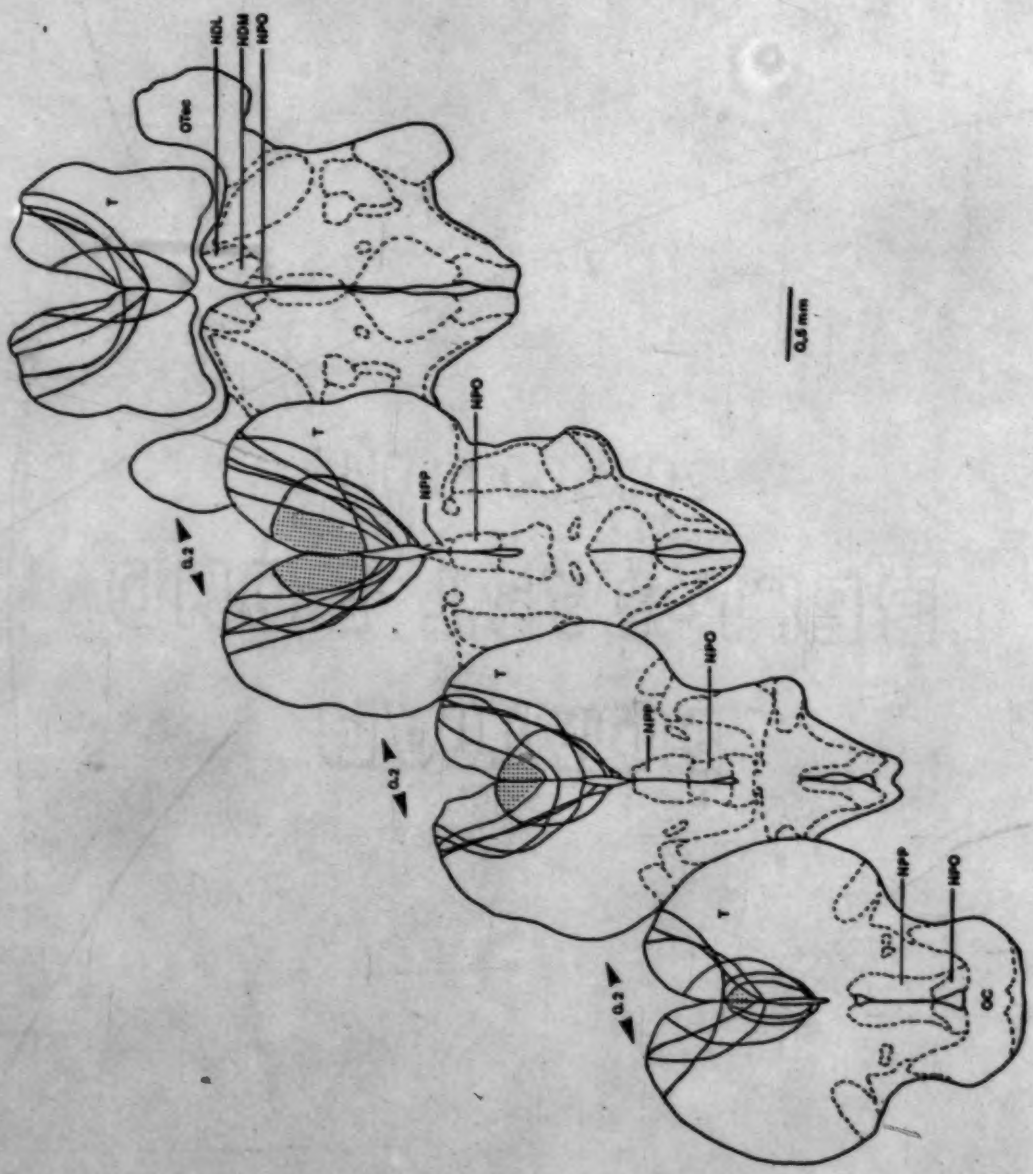

OHN
NDH
TDH
OTec
I
C
NPP
0,2
0,5 mm
OC

Figure 29. A diagram summarizing lesioned areas of the posterior hypothalamus of goldfish subjected to a thermal stress

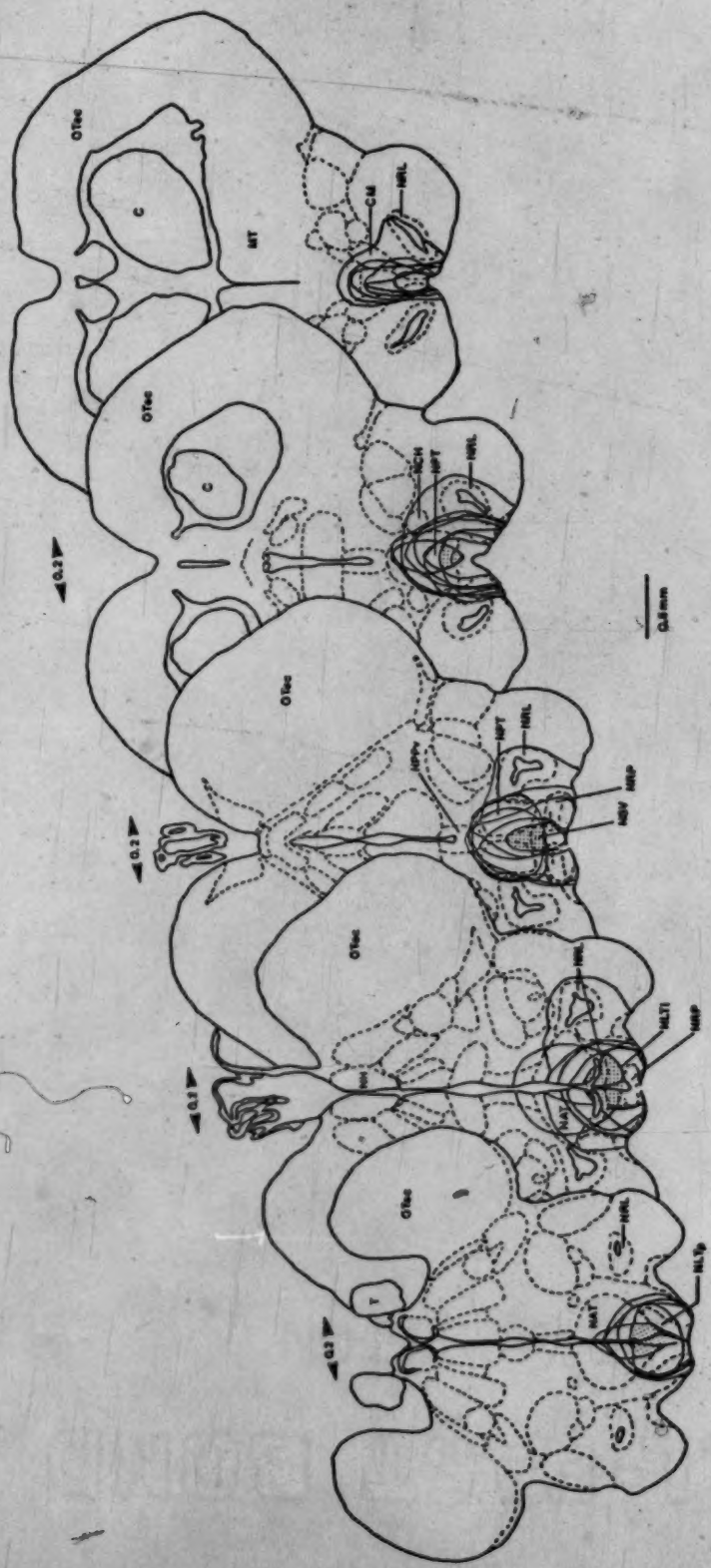
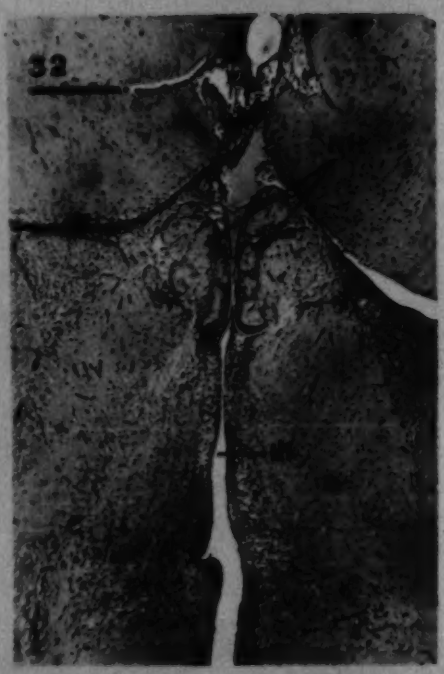

OTec
MT
CM
HRL
HPT
HRV
HRP
HLTI
HLTp
C1
C2
C3
0.5 mm

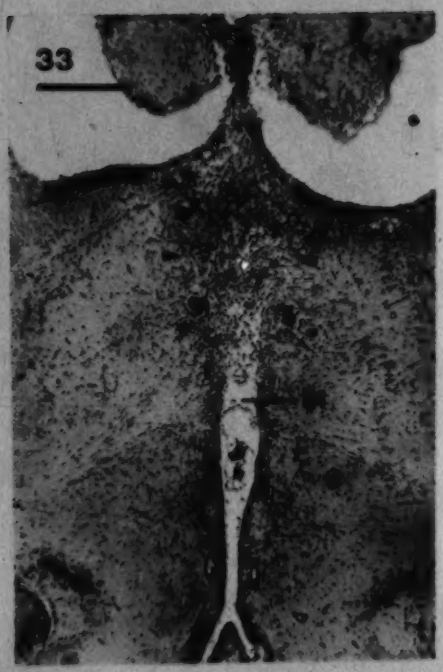
Figure 30. A cross-section through the brain of a goldfish bearing a bilateral lesion (arrows) of the dorsal telencephalon. Scale 200 µm.

Figure 31. A cross-section through the brain of a goldfish bearing a lesion (arrows) in the posterior hypothalamus. Scale 200 µm.

Figure 32. A cross-section through the brain of a non-lesioned goldfish showing the habenular nuclei. Scale 200 µm.

Figure 33. A cross-section through the brain of a goldfish bearing a lesion (arrows) of the habenular nuclei. Scale 200 µm.


32


33

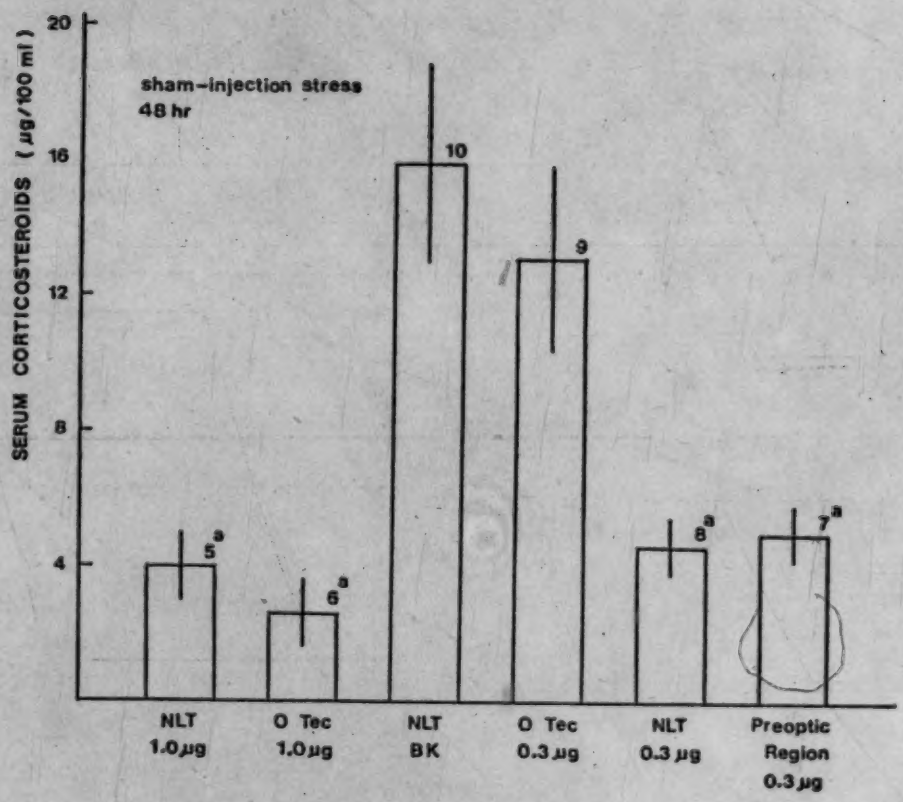
cortisol or a blank pellet into various regions of the brain are shown in Figure 34. Goldfish with pellets containing approximately 1.0 µg cortisol implanted in the optic tectum or the NLT had serum corticosteroid levels which were significantly lower ($p < 0.001$) than in goldfish with blank pellet implants in the NLT or goldfish with pellets containing approximately 0.3 µg cortisol in the optic tectum. The serum corticosteroid levels of goldfish implanted with pellets containing approximately 0.3 µg cortisol in the NLT or preoptic region were significantly lower ($p < 0.001$) than in goldfish with blank pellet implants in the NLT or in goldfish implanted with pellets containing approximately 0.3 µg cortisol in the optic tectum.

A diagram of the goldfish brain illustrating the implant sites for the 0.3 µg cortisol pellets in the NLT and the preoptic region is shown in Figure 35. A cross-section through the brain of a goldfish bearing a cortisol pellet implant in the NLTa is shown in Figure 36. Cortisol pellets implanted in the rostral NLT were located in the third ventricle, making intimate contact with the cells of the NLTa or the rostral NLTp. Examples of cortisol pellet implant sites in the preoptic region are presented in Figure 37 and Figure 38. Cortisol pellets implanted in the preoptic region were located in the preoptic recess of the third ventricle. Five of these pellets made contact with the neurosecretory cells of the NPO and two pellets were located just dorsal to the NPO.

The serum corticosteroid levels of goldfish in response to a sham-injection stress at 48 hours after the implantation of a blank pellet in the NLT or the pituitary gland, or a pellet containing

Figure 34. Serum corticosteroid levels (mean ± SEM) in response to a sham-injection stress of goldfish bearing pellet implants containing approximately 1.0 µg cortisol in the NLT or optic tectum (O Tec), 0.3 µg cortisol in the NLT, optic tectum, or preoptic region, or a blank pellet in the NLT. Numbers denote the number of fish per implant site.

a, significantly lower ($p < 0.001$) than goldfish bearing a blank pellet implant in the NLT or a 0.3 µg cortisol pellet in the optic tectum.


SERUM CORTICOSTEROIDS (µg/100 ml)
20
16
12
8
4
sham-injection stress
48 hr
10
9
5a
6a
8a
7a
NLT 1.0µg
O Tec 1.0µg
NLT BK
O Tec 0.3µg
NLT 0.3µg
Preoptic Region 0.3µg

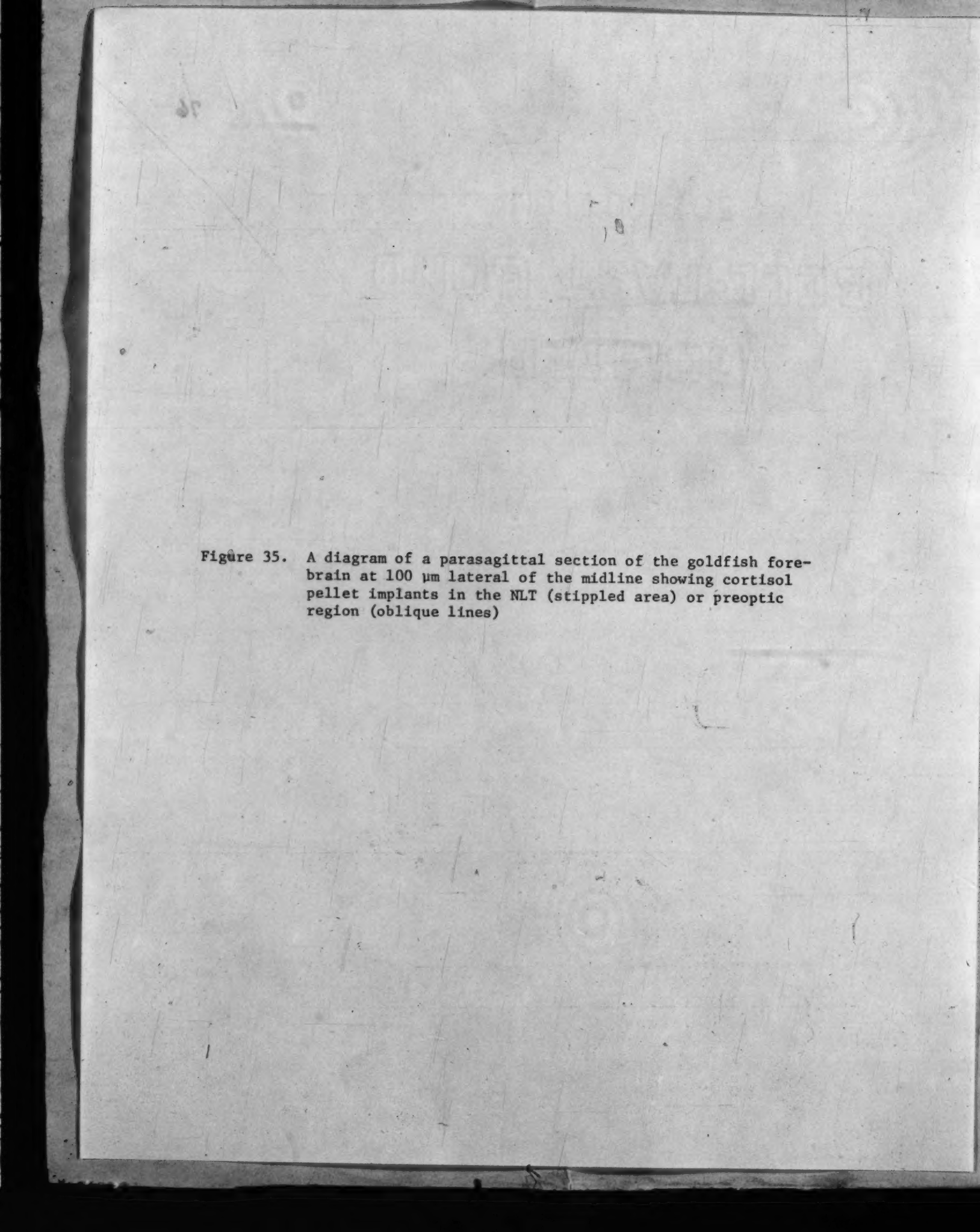


Figure 35. A diagram of a parasagittal section of the goldfish forebrain at 100 µm lateral of the midline showing cortisol pellet implants in the NLT (stippled area) or preoptic region (oblique lines)

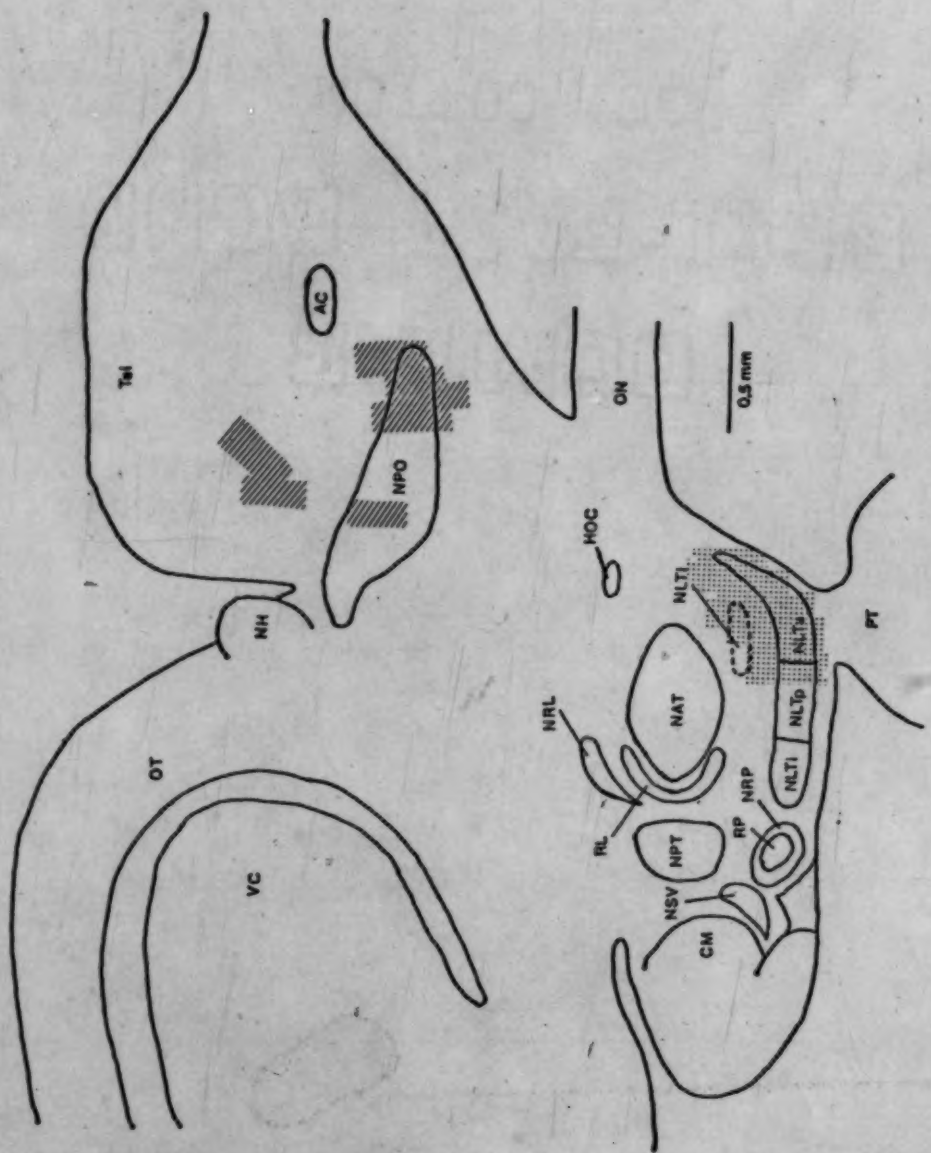
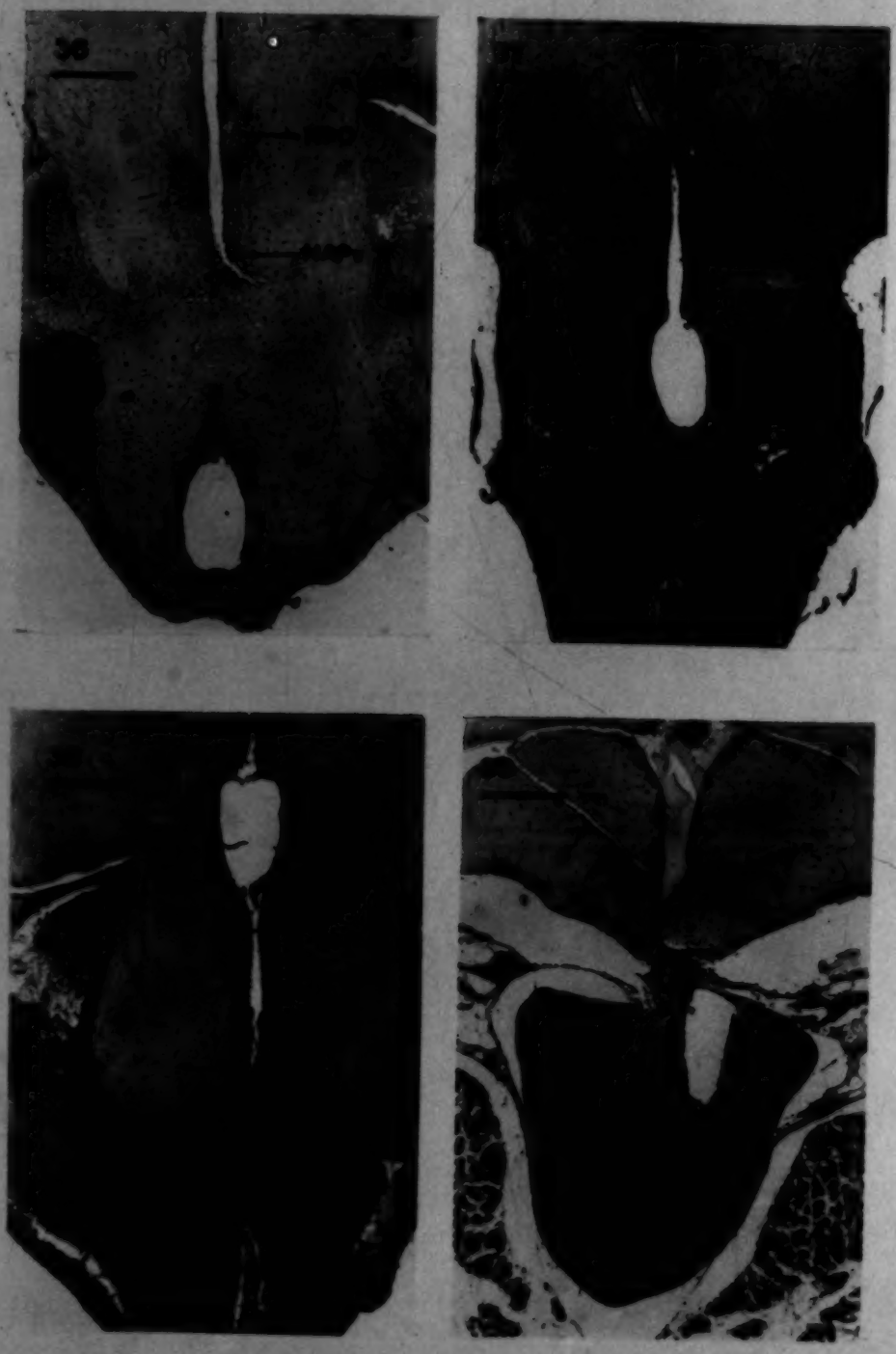

AC
Tel
NPO
NH
OT
VC
HOC
ON
0.5 mm
NLTi
NLTa
NLTp
PT
NRL
NAT
RL
NPT
RP
NRP
NSV
CM

Figure 36. A cross-section through the brain of a goldfish bearing a pellet (arrows) containing approximately 0.3 µg cortisol in the NLTa. Scale 200 µm.

Figures 37 and 38. Cross-sections through the brains of two goldfish bearing pellets (arrows) containing approximately 0.3 µg cortisol in the preoptic recess of the third ventricle. Scale 200 µm.

Figure 39. A cross-section through the brain and pituitary of a goldfish bearing a pellet (arrows) containing approximately 0.5 µg cortisol in the pituitary. Scale 200 µm.


36

approximately 0.5 µg cortisol in the optic tectum, NLT, or pituitary gland are shown in Figure 40. Goldfish bearing a 0.5 µg cortisol pellet implant in the optic tectum had serum corticosteroid levels which were not significantly different from goldfish bearing a blank pellet implant in the NLT or the pituitary. The serum corticosteroid concentrations of goldfish with a 0.5 µg cortisol pellet implanted in the NLT were significantly lower ($p < 0.001$) than those observed in goldfish with a blank pellet implant in the NLT or a 0.5 µg cortisol pellet in the optic tectum. Goldfish with pellets containing 0.5 µg cortisol implanted in the pituitary gland had serum corticosteroid levels which were not significantly different from fish bearing blank pellet implants in the pituitary. A section through the pituitary of a goldfish bearing a cortisol pellet implanted in the pars distalis is shown in Figure 39. A diagram illustrating the implant sites in the NLT is shown in Figure 41. The corticosteroid-containing pellets were situated in the third ventricle making contact with the cells of the NLTa and rostral NLTp.

The serum corticosteroid concentrations of goldfish in response to a sham-injection stress 48 hours after implantation of a pellet containing approximately 0.5 µg cortisol in the NLT, dorsomedial hypothalamus, posterior hypothalamus, or preoptic region are shown in Figure 42. Goldfish with 0.5 µg cortisol pellet implants in the dorsomedial hypothalamus or posterior hypothalamus had corticosteroid levels that were similar to goldfish bearing a blank pellet implant in the posterior hypothalamus. The serum corticosteroid concentrations of goldfish with a 0.5 µg cortisol pellet implant in the NLT or preoptic region were significantly lower ($p < 0.001$) than in goldfish bearing cortisol pellets

Figure 40. Serum corticosteroid levels (mean ± SEM) of goldfish in response to a sham-injection stress at 48 hours post-implantation of a pellet containing approximately 0.5 µg cortisol in the optic tectum (O Tec), NLT, or pituitary (Pit), or a blank pellet in the NLT or pituitary. Numbers indicate the number of fish per implant site.

a, significantly lower ($p < 0.001$) than goldfish bearing a blank pellet implant in the NLT.

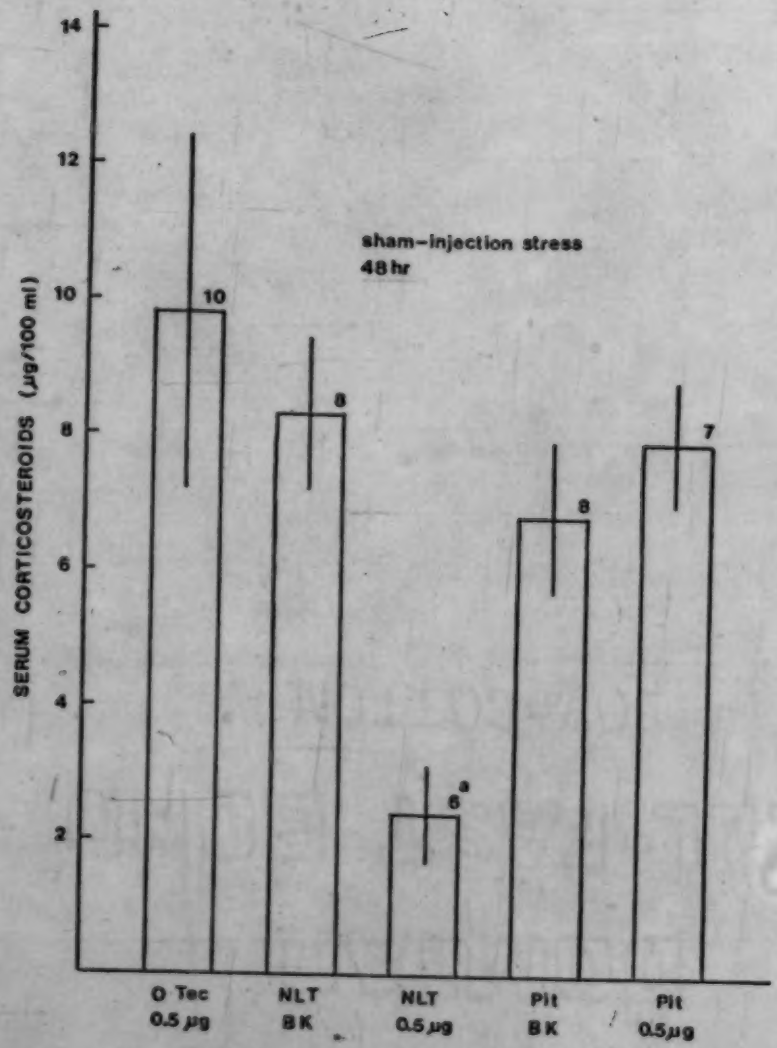

sham-injection stress
48 hr
SERUM CORTICOSTEROIDS (µg/100 ml)
14
12
10
8
6
4
2
10
8
6a
8
7
O Tec 0.5 µg
NLT BK
NLT 0.5 µg
Pit BK
Pit 0.5 µg

Figure 41. A diagram of a parasagittal section of the goldfish forebrain 100 µm lateral of the midline showing cortisol pellet implants in the NLT (stippled area)

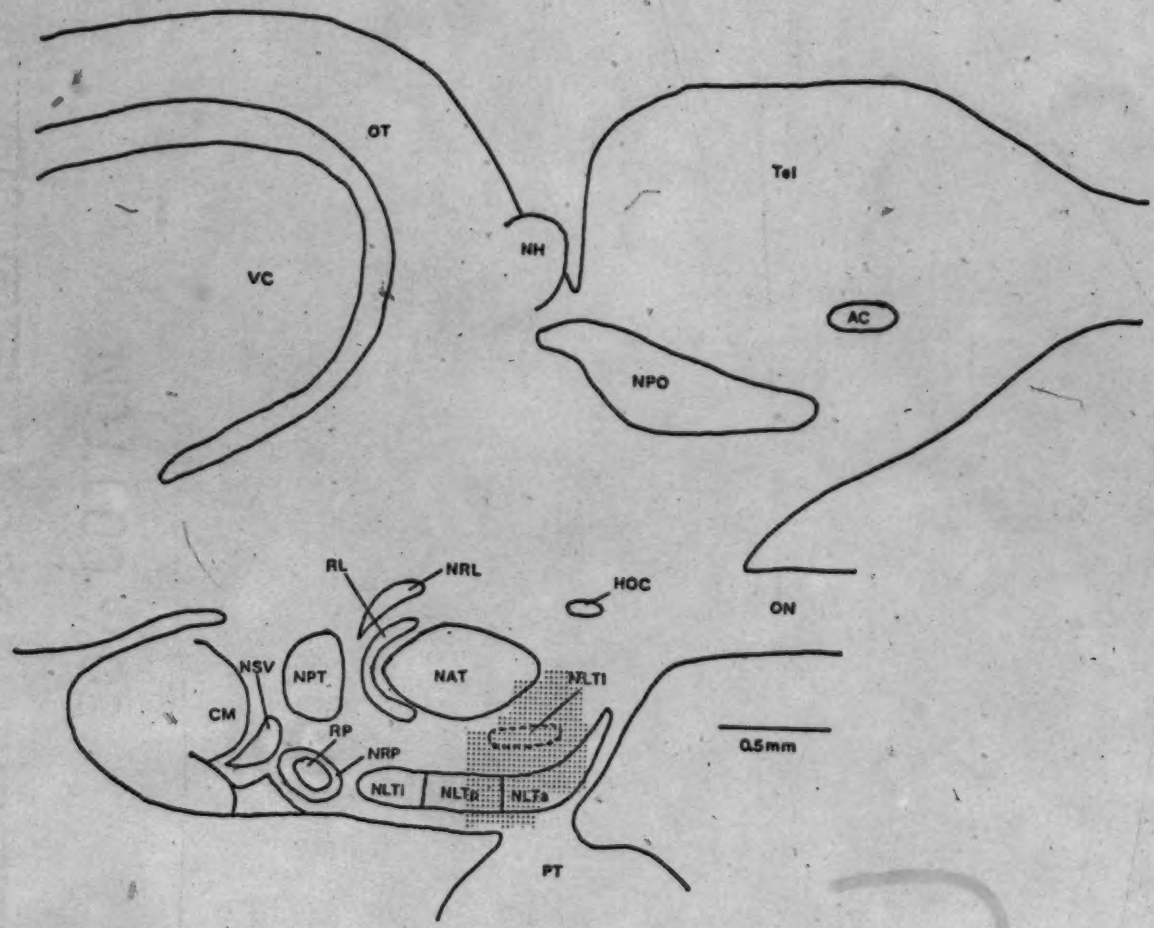
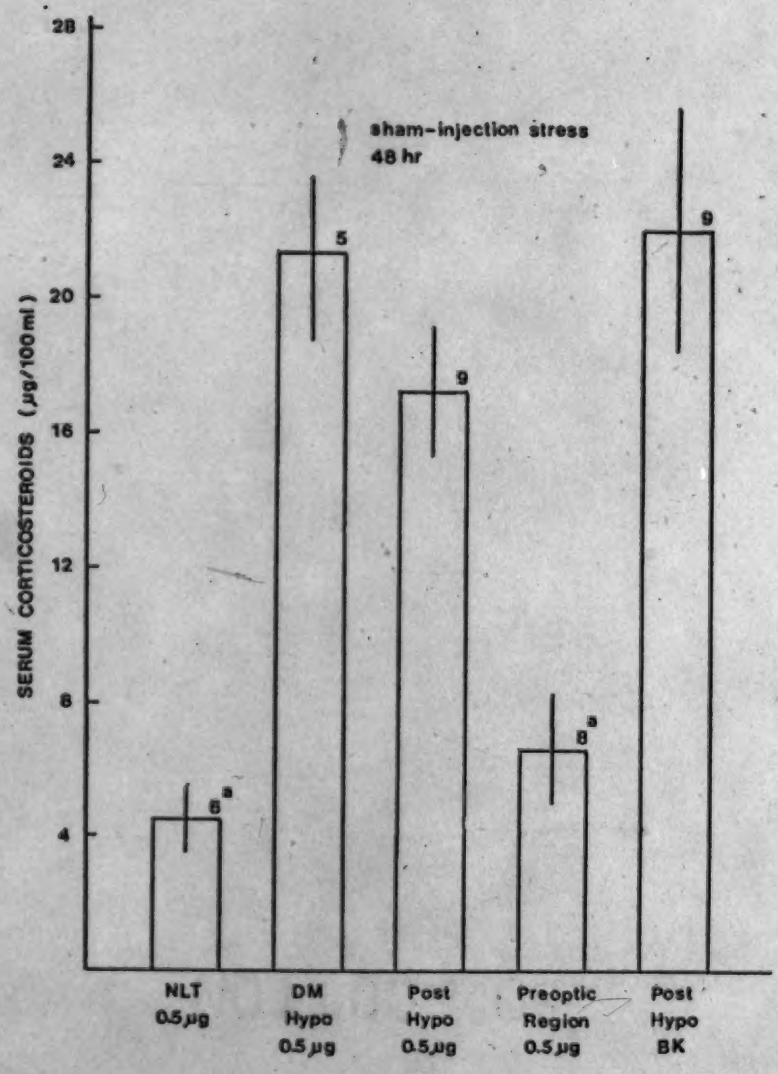

OT
VC
NH
Tel
AC
NPO
RL
NRL
HOC
ON
NSV
NPT
NAT
NLTI
CM
RP
NRP
NLTi
NLTp
NLTa
0.5mm
PT

Figure 42. Serum corticosteroid levels (mean ± SEM) of goldfish in response to a sham-injection stress at 48 hours post-implantation of pellets containing approximately 0.5 µg cortisol in the NLT, dorsomedial hypothalamus (DM Hypo), posterior hypothalamus (Post Hypo) or preoptic region, or a blank pellet in the posterior hypothalamus. Numbers indicate the number of fish in each group.

a, significantly lower ($p < 0.001$) than goldfish bearing a blank pellet implant in the posterior hypothalamus or a 0.5 µg cortisol pellet in the dorsomedial hypothalamus or posterior hypothalamus.


sham-injection stress
48 hr
SERUM CORTICOSTEROIDS (µg/100ml)
28
24
20
16
12
8
4
6
5
9
8
9
NLT
0.5µg
DM
Hypo
0.5µg
Post
Hypo
0.5µg
Preoptic
Region
0.5µg
Post
Hypo
BK

in the dorsomedial hypothalamus or posterior hypothalamus, or blank pellets in the posterior hypothalamus. A diagram of the goldfish brain illustrating the implant sites in the dorsomedial hypothalamus, posterior hypothalamus, NLT, and preoptic region is presented in Figure 43. Figure 47 shows a section through the brain of goldfish bearing a cortisol pellet implant in the dorsomedial hypothalamus. Cortisol pellet implant sites in the dorsomedial hypothalamus were located immediately posterior to the horizontal commissure. A section through the brain of a goldfish with a cortisol pellet implant in the posterior hypothalamus is shown in Figure 48. Implants in the posterior hypothalamus extended from the lateral recess of the third ventricle to just anterior of the corpus mamillare.

The serum corticosteroid levels of goldfish in response to a sham-injection stress at 48 hours after the implantation of either a blank pellet into the preoptic recess of the third ventricle or pellets containing approximately 0.5 µg cortisol implanted into various areas of the telencephalon are shown in Figure 44. Goldfish bearing cortisol pellets implanted unilaterally, approximately 700 µm from the midline in the telencephalon rostral to the anterior commissure had corticosteroid levels which were not significantly different from goldfish bearing a blank pellet implant in the telencephalon. Cortisol pellets implanted unilaterally in the telencephalon approximately 700 µm from the midline posterior to the anterior commissure, or implanted into the preoptic recess of the third ventricle resulted in serum corticosteroid levels which were significantly lower ($p < 0.025$) than blank pellet implants in the telencephalon. A diagram illustrating the unilateral implant sites

Figure 43. A diagram of a parasagittal section of the goldfish forebrain 100 µm lateral of the midline showing cortisol pellet implants in the NLT (stippled area), preoptic region (oblique lines), posterior hypothalamus (horizontal lines) or dorsomedial hypothalamus (vertical lines)

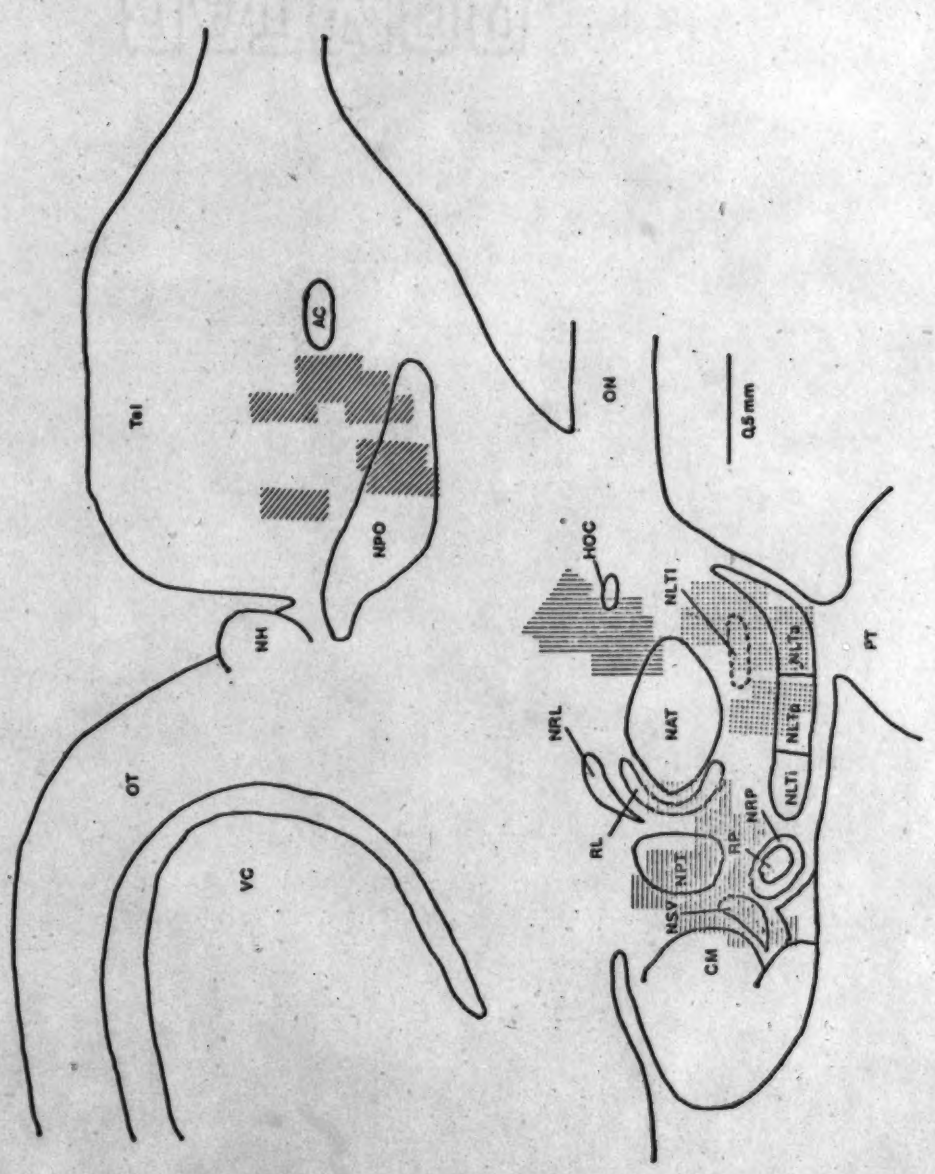
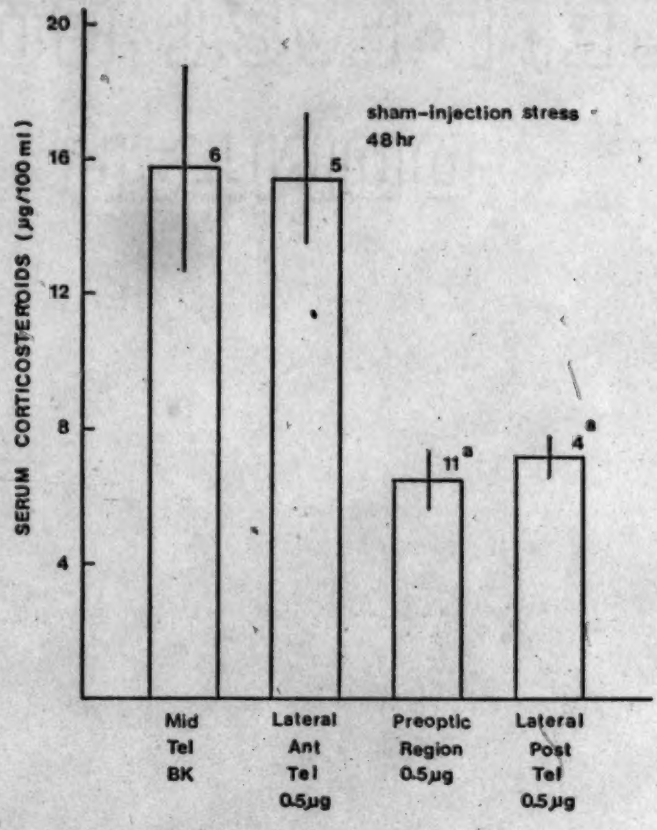

AC
Tel
NPO
NH
OT
VC
ON
0.5 mm
HOC
NLTi
NLTp
NLTa
PT
NRL
NAT
RL
NRP
RP
NPE
NSP
CM

Figure 44. Serum corticosteroid levels (mean ± SEM) of goldfish in response to a sham-injection stress at 48 hours post-implantation of pellets containing approximately 0.5 µg cortisol in the lateral anterior telencephalon, preoptic region, lateral posterior telencephalon or a blank pellet in the preoptic region. Numbers indicate the number of fish in each group.

a, significantly lower ($p < 0.025$) than goldfish bearing a blank pellet implant in the preoptic region or a pellet containing 0.5 µg cortisol in the lateral anterior telencephalon.


SERUM CORTICOSTEROIDS (µg/100 ml)
20
16
12
8
4
sham-injection stress
48 hr
6
5
11a
4a
Mid Tel BK
Lateral Ant Tel 0.5µg
Preoptic Region 0.5µg
Lateral Post Tel 0.5µg

in the telencephalon is presented in Figure 45. A section through the brain of a goldfish with a unilateral cortisol pellet implant in the telencephalon rostral to the anterior commissure is shown in Figure 49. The cortisol pellet implant sites in the preoptic recess of the third ventricle and in the midline anterior the anterior commissure are illustrated in Figure 46.

Attempts to implant cortisol pellets in the midline, anterior to the anterior commissure met with minimal success. Two goldfish with cortisol implants in this area had serum cortisol levels of 18.3 and 16.0 µg/100 ml. Figure 50 contains a section through the brain of a goldfish bearing a unilateral cortisol pellet implant in the lateral posterior telencephalon.

Figure 45. Unilateral implants of pellets containing approximately 0.5 µg cortisol in the goldfish forebrain at about 700 µm lateral to the midline in the anterior telencephalon (stippled area) or posterior telencephalon (oblique lines), shown on a diagram of a parasagittal section 100 µm lateral of the midline

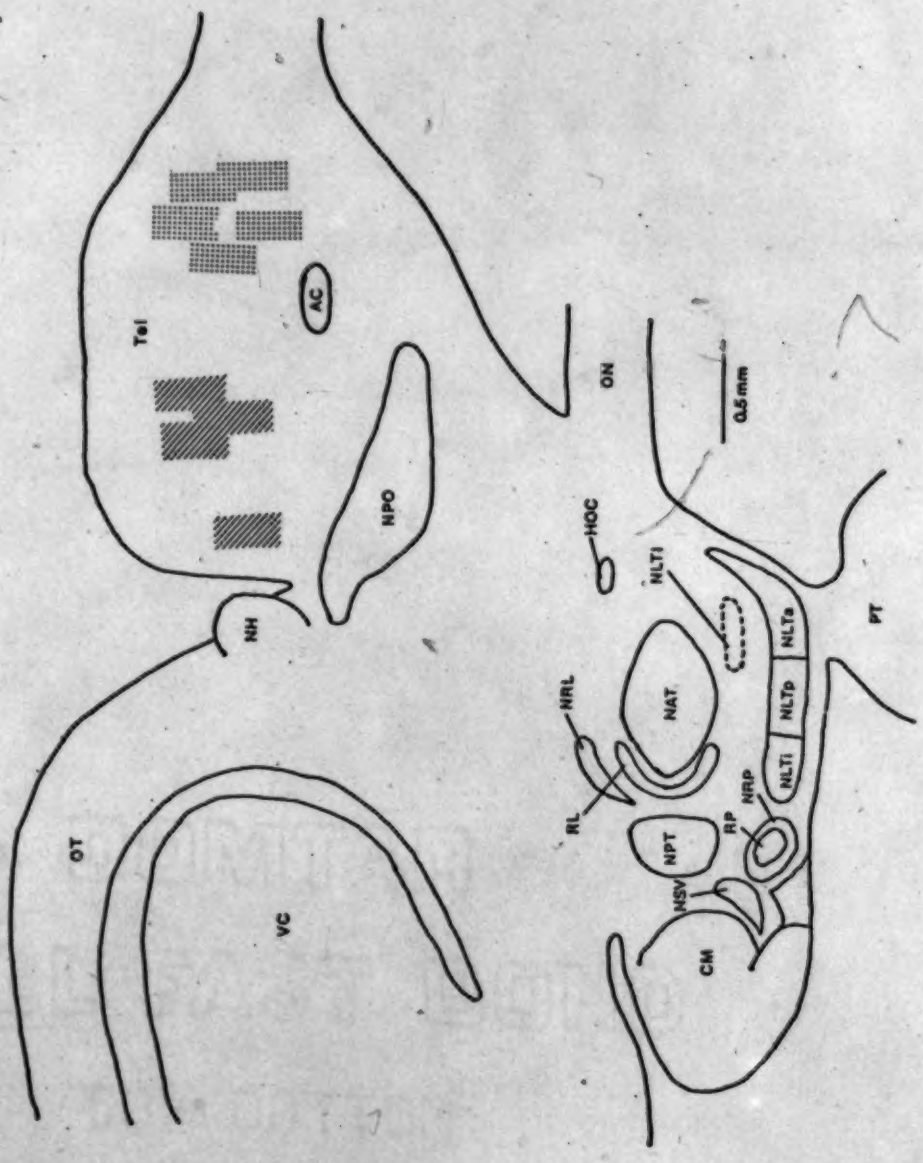

Tel
AC
NPO
NH
OT
VC
ON
0.5 mm
HOC
NLTi
NLTa
NLTp
NLTi
PT
NRL
NAT
RL
NRP
RP
NPT
NSV
CM

Figure 46. A diagram of a parasagittal section of the goldfish forebrain 100 µm lateral of the midline showing cortisol pellet implants in the preoptic region (oblique lines) or anterior telencephalon (stippled area)

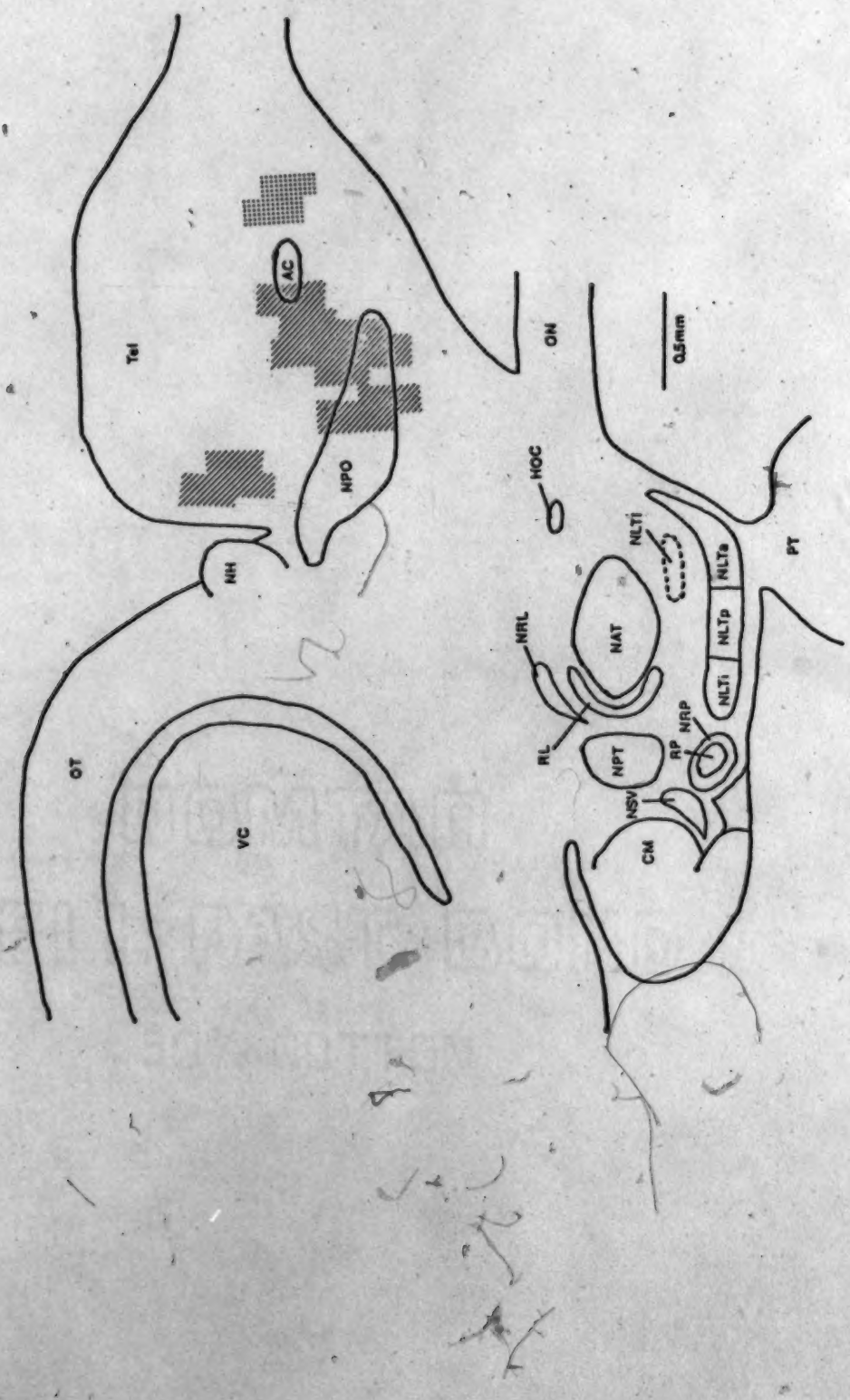
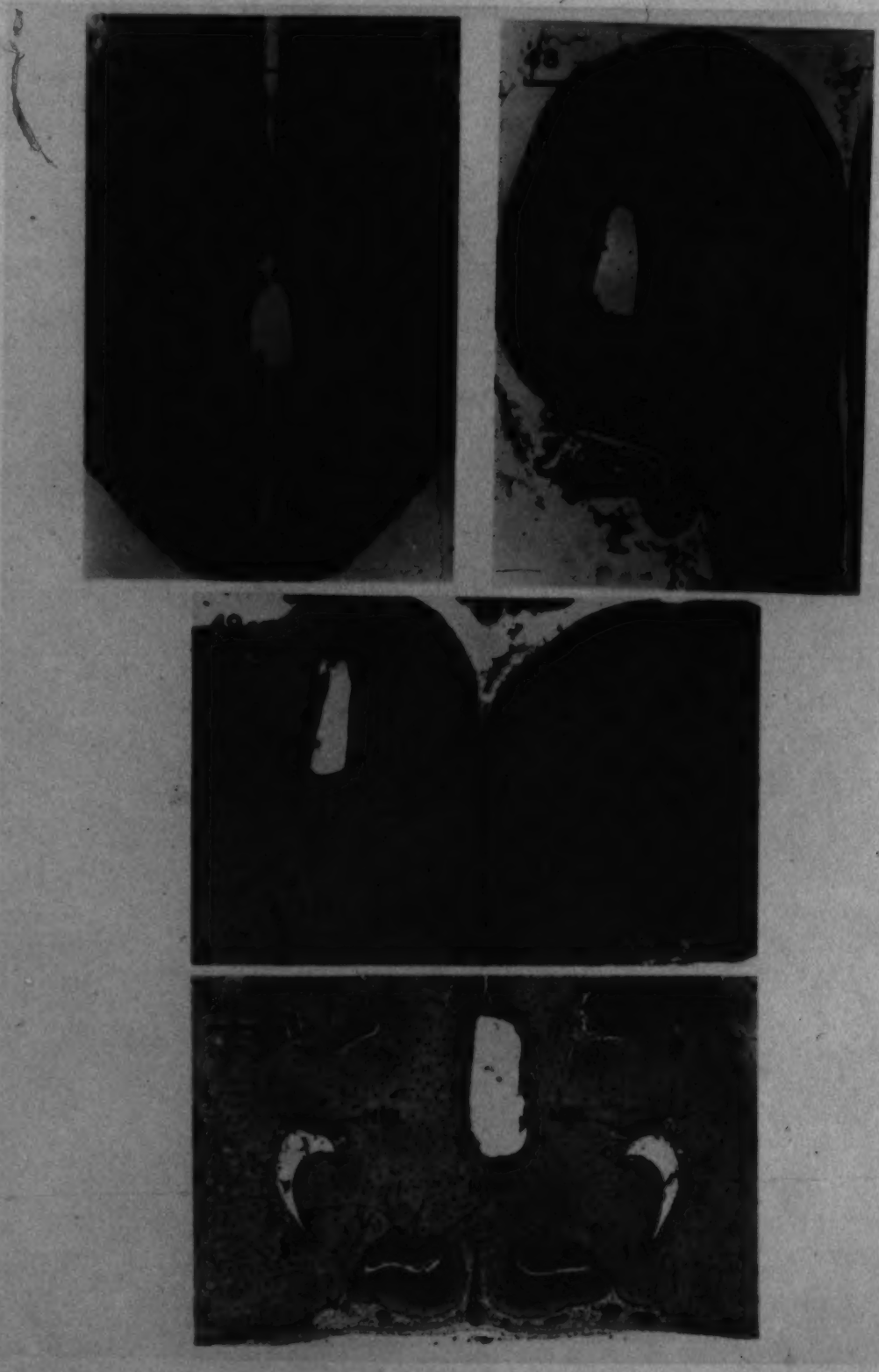

Tel
AC
NPO
NH
OT
VC
ON
0.5mm
HOC
NLTi
NRL
NAT
NLTo
NLTp
PT
NLTi
NRP
RL
RP
NPT
NSV
CM

Figure 47. A cross-section through the brain of a goldfish bearing a pellet implant (arrows) containing approximately 0.5 µg cortisol in the dorsomedial hypothalamus. Scale 200 µm.

Figure 48. A cross-section through the brain of a goldfish bearing a pellet (arrows) containing approximately 0.5 µg cortisol in the posterior hypothalamus. Scale 200 µm.

Figure 49. A cross-section through the brain of a goldfish bearing a pellet implant (arrows) containing approximately 0.5 µg cortisol in the lateral telencephalon anterior to the anterior commissure. Scale 200 µm.

Figure 50. A cross-section through the brain of a goldfish bearing a pellet implant (arrows) containing approximately 0.5 µg cortisol in the lateral mid-telencephalon. Scale 200 µm.



DISCUSSION

I. BRAIN TISSUE EXTRACTS EXPERIMENTS

In the present investigations, the injection of lyophilized acid extracts of longnose sucker, or goldfish, hypothalamus or telencephalon into betamethasone-blocked goldfish resulted in serum corticosteroid concentrations that were significantly greater than those observed following saline injection, or injection of extracts of the cerebellum. These results provide evidence for the presence of a CRF in sucker and goldfish hypothalamus and telencephalon.

The results obtained following the injection of hypothalamic extract material prepared from suckers collected in 1973 suggest a dose-response relationship. However, the corticosteroid levels observed in goldfish receiving a dose of extract equivalent to 18.75 hypothalami were not significantly greater than those observed in goldfish receiving a dose of extract equivalent to 6.25 hypothalami. Thus, a significant dose-response relationship was not established. Furthermore, a dose-response relationship was not evident in the response to the hypothalamic extract material prepared from suckers collected in 1975 or in the goldfish hypothalamic extract material. The results obtained following the injection of telencephalon extract material prepared from goldfish suggest a dose-response relationship; however, due to the large individual variation in response to the extract material, no significant differences were observed between the different dosages used. A dose-response relationship was not evident in the extract material prepared

from the telencephalon of suckers collected in 1975. Further work is required to establish a dose-response relationship for the CRF activity in extracts of the sucker and goldfish hypothalamus and telencephalon.

A daily rhythm in CRF activity has been shown to exist in the hypothalamus of the rat (Hiroshige *et al.*, 1969) and the pigeon (Sato and George, 1973). In the present study, the brain tissue samples were collected from both the suckers and the goldfish over an extended period of time during the day. This may have resulted in differences in the amount of CRF activity in the hypothalami and telencephalons collected. Furthermore, CRF activity in the rat hypothalamus has been shown to increase in response to stress (Takabe *et al.*, 1972). The goldfish from which brain tissues were collected in this study were living in large holding tanks in captivity, whereas the suckers were captured on their spring upstream spawning migration in swift flowing water. In addition to the stress of the spawning migration, these suckers were further stressed by being held in fish boxes for periods of time up to one-half hour before they were killed. These vast differences in living and collecting conditions could very well affect the CRF activity present in the hypothalamus. In this regard, it would be interesting to determine if teleosts respond to stress with increases in the content of CRF in the hypothalamus. In addition, the amount of brain tissue extracted was different for the goldfish and the suckers. The total wet weight of the hypothalamus, telencephalon, and cerebellum fragments collected from the suckers was approximately twice the wet weight of the same tissues collected from the same number of goldfish. Thus, a comparison of the relative potency of the extracts prepared from the goldfish and sucker hypothalami or telencephalons is not justified.

Sage and Purrott (1969) made the first report of CRF activity in extracts of fish brain tissue. They observed an increase in the spontaneous release of ACTH from cultured goldfish pituitaries when a crude hypothalamic extract was added to the incubation medium.

Previous attempts to demonstrate CRF activity in teleost brain tissue extracts in an *in vivo* test system have been unsuccessful. Hawkins and Ball (1970) found that crude saline homogenates of goldfish hypothalamus or telencephalon were ineffective in elevating plasma cortisol levels of mollies bearing pituitary autotransplants. In an extension of this work, Hawkins and Ball (1973) reported that lyophilized acid extracts of rat median eminence were potent stimulators of cortisol secretion in mollies bearing pituitary autotransplants. According to Chan *et al.* (1969) rat median eminence extracts contain potent CRF activity when tested on rat pituitaries *in vitro*. However, this stimulation of cortisol secretion in the molly by rat median eminence extract was due, in part, to a direct effect on the interrenal gland, as the extracts also stimulated cortisol secretion in hypophysectomized fish. Hawkins and Ball attributed this effect to the presence of ACTH and/or an ACTH mimic in the extract material.

In additional experiments, Hawkins and Ball (1973) employed dexamethasone-blocked mollies to assay CRF activity in brain extracts of the same species. Injection of crude saline homogenates of hypothalamus or telencephalon did not stimulate cortisol secretion. The pituitary-interrenal axis of such fish was activated by lyophilized acid extracts of rat median eminence tissue. However, saline extracts of rat median eminence did not stimulate cortisol secretion when tested at the same

dose as the lyophilized acid extract material (Hawkins, unpublished data reported in Hawkins and Ball, 1973). Unfortunately, lyophilized acid extracts of the hypothalamus or telencephalon of mollies or goldfish were not tested for their ability to stimulate cortisol secretion in the dexamethasone-blocked mollies. According to the present results, CRF activity is preserved by acid extraction.

Hawkins and Ball (1973) reported that arginine vasopressin was a potent stimulator of cortisol secretion in mollies bearing pituitary autotransplants. Arginine vasopressin, on the other hand, was ineffective in stimulating cortisol secretion in hypophysectomized mollies. This suggests that the action of this peptide in stimulating cortisol secretion in mollies bearing pituitary autotransplants occurred by a release of ACTH from the pituitary gland. However, the physiological significance of arginine vasopressin stimulating ACTH secretion in the molly may be of pharmacological interest only as teleosts do not synthesize arginine vasopressin.

In the teleost brain the preoptic nucleus extends well into the lobes of the telencephalon. In teleosts the preoptic nucleus is the site of the production of the neurohypophysial octapeptide hormones, arginine vasotocin and isotocin (Perks, 1969). In mammals the neurohypophysial peptide arginine vasopressin has potent CRF activity both *in vivo* and *in vitro* (for a review see Yates and Maran, 1974). Hawkins and Ball (1973) demonstrated that arginine vasopressin stimulated the pituitary-interrenal axis of mollies bearing pituitary autotransplants. This suggests that the CRF activity observed in extracts of the goldfish and sucker telencephalon in the present investigation may have been due

to the presence of arginine vasotocin and/or isotocin. In this regard, studies of the potency of these octapeptides in stimulating ACTH secretion in teleosts would be of particular interest.

The ability of lyophilized acid extracts of sucker or goldfish hypothalami to stimulate cortisol secretion in betamethasone-blocked goldfish suggests the presence of a hypothalamic CRF. The possibility that the hypothalamic extract material may have contained ACTH which could stimulate the interrenal directly should not be totally excluded as the extracts were not tested to see if they could stimulate corticosteroid secretion in hypophysectomized goldfish. However, such activity should be destroyed by boiling in the extraction procedure. Also, it is noteworthy that Sage and Purrott (1969) could not detect ACTH activity in saline homogenates of goldfish hypothalamus, indicating that the goldfish hypothalamus does not contain appreciable ACTH.

The nature of the CRF present in the extracts prepared from the goldfish and sucker hypothalamus is not known. Although CRF was the first hypothalamic hormone to be demonstrated in mammals (Saffran and Schally, 1955), its instability has delayed its isolation and the elucidation of its structure. However, it appears to be a polypeptide since it is destroyed by some proteolytic enzymes (Schally *et al.*, 1968). Literature speculating on the structure of CRF has been summarized in a review by Schally *et al.* (1973). In view of the fact that extracts of the rat median eminence stimulate ACTH secretion in the molly (Hawkins and Ball, 1973) it would be particularly interesting to investigate the possibility that extracts of the goldfish or sucker hypothalamus might stimulate ACTH secretion in a mammal such as the rat.

II. HYPOTHALAMIC LESION EXPERIMENTS

The effects of lesions in the NLT provide direct evidence for hypothalamic control of ACTH secretion in the goldfish. Lesions which destroyed the NLTa and rostral NLTp suppressed stress-induced increases in circulating levels of adrenocorticosteroids. Smaller lesions in either the NLTa or NLTp failed to suppress this stress response. Lesions of the NLTi extending into the posterior hypothalamus and lesions placed immediately dorsal to the NLT also failed to suppress the stress response. The NLTl does not appear to play a role in the control of ACTH secretion as lesions of the NLTa and rostral NLTp were equally as effective in suppressing the stress response as larger lesions of the NLT which included the NLTa, NLTp, and NLTl. These findings therefore indicate that the NLTa and rostral NLTp are the source of a factor that stimulates ACTH secretion in the goldfish. This factor may be CRF, shown to exist in the hypothalamus of goldfish and longnose sucker in the extract experiments discussed above.

Large lesions of the NLT in the area which suppressed the stress response of goldfish had no effect on non-stress levels of serum corticosteroids. The failure of these lesions to alter non-stress levels of corticosteroids may reflect some degree of autonomy of ACTH secretion by the pituitary and/or interrenal gland activity independent of ACTH stimulation. Recent studies by Porthé-Nibelle and Lahlou (1974) support the latter hypothesis. They found that, following hypophysectomy, circulating levels of corticosteroids were not significantly different from those observed with intact goldfish. This finding indicates that the goldfish interrenal possesses sufficient autonomy to maintain basal

levels of corticosteroids independent of ACTH secretion.

The NLT has been identified as an important hypophysiotrophic area in the goldfish. Lesioning studies of the NLT have shown that the NLTa and NLTp are the source of an inhibitory factor for thyrotrophin stimulating hormone (Peter, 1970). Lesions of the NLTp and posterior portion of the NLTa have indicated that this area of the hypothalamus is the source of gonadotrophin releasing factor (Peter, 1970). Lesion studies which destroyed the NLTl have demonstrated that this area is the source of an inhibitory factor controlling the release of prolactin from the pituitary gland (Peter and McKeown, 1974). The results of the lesion experiments in the present investigation provide direct evidence for the NLT controlling the secretion of an additional pituitary hormone, ACTH.

Lesioning studies have been employed to identify areas of the hypothalamus responsible for the stimulation of ACTH secretion in mammals, birds, reptiles, and amphibians. In mammals, lesions of the tuberal region of the hypothalamus and the median eminence region block stress-induced increases in ACTH secretion. Brodish (1963) reported on the effects of hypothalamic lesions on ACTH secretion in the rat. He concluded that there was a diffuse hypothalamic network responsible for the control of ACTH secretion, extending over the length of the medial basal hypothalamus from the optic chiasma to the mamillary bodies. The impairment of ACTH release was proportional to the amount of tissue destroyed, as small lesions throughout this area were effective. However, lesions in the median eminence-tuberal region were most effective. In contrast to the diffuse hypothalamic network involved in the control of ACTH secretion in the rat, the results of the lesioning studies in

the present investigation suggest that a relatively smaller though homologous region in the medial basal hypothalamus is involved in the control of ACTH secretion in the goldfish.

In birds, the corticotrophic area of the hypothalamus seems to be localized within a more defined region than that oberved in the rat. Lesions of the posterior mediolateral hypothalamic region in the pigeon reduce basal corticosteroid concentrations and stress-induced increments in plasma corticosteroid levels (Baylé and Bouillé, 1971; Bouillé and Baylé, 1973). In a lizard, *Sceloporus cyanogenys*, lesions placed in the midline of the anterior basal hypothalamus and in the median eminence reduced adrenal gland weight, indicating this to be a corticotrophic area in the reptilian hypothalamus (Callard *et al.*, 1973). In an anuran, *Bufo bufo*, transections of the brain stem immediately rostral to the median eminence or immediately caudal to the optic chiasm reduced plasma corticosterone concentrations (Büchmann *et al.*, 1972). Transections of the brain anterior to the optic chiasm did not affect plasma corticosteroid levels. These results indicate a hypothalamic involvement in the control of corticotrophic function in amphibians. In the present investigation, lesions of the NLT in the mediobasal hypothalamus suppressed the stress response of goldfish. These results indicate that in all of the vertebrate classes a generally homologous region of the hypothalamus is involved in the stimulation of ACTH secretion.

In the present study, large lesions of the NPO suppressed stress-induced increases in circulating levels of adrenocorticosteroids. Small lesions of the NPO failed to suppress the stress response. In the European eel, Leatherland and Dodd (1969) noted a depletion of neurosecretory material from the neurohypophyseal system in response to

non-specific stressors such as electric fishing, temperature shocks and abrupt salinity changes. Hawkins and Ball (1973) found in both betamethasone-blocked mollies and mollies bearing pituitary auto-transplants, that intraperitoneal injections of arginine vasopressin significantly stimulated corticosteroid secretion, suggesting that neuro-hypophyseal peptides may play a role in stimulating ACTH secretion in teleosts. This is further supported by the findings in the present study that extracts of the telencephalon, which include tissues of the NOP, possess CRF activity. The strongest evidence for a physiological role of neurohypophyseal peptides in ACTH secretion in teleosts comes from the demonstration, in the present investigations, that goldfish bearing lesions of the NPO exhibit an impairment in ACTH secretion in response to stress.

Neurohypophyseal peptides have been shown to stimulate ACTH secretion in amphibians (Jorgensen, 1965) and mammals (for a review see Yates and Maran, 1974). In birds, however, neurohypophyseal hormones are ineffective in stimulating ACTH release (Sato and George, 1973) and are considered to play an insignificant role in ACTH secretion (Frankel *et al.*, 1967). In mammals, it is now accepted that vasopressin has CRF activity. Vasopressin and synthetic analogues of vasopressin have been shown to release ACTH *in vivo* when given systemically (for a review see Yates and Maran, 1974) and when injected directly into the pituitary of unanesthetized dogs in doses which were systemically ineffective (Gonzalez-Luque *et al.*, 1970). Vasopressin and synthetic analogues of vasopressin also stimulate ACTH release from pituitary tissue incubated *in vitro* (Fleischer and Rawls, 1970; Pearlmutter *et al.*, 1974). Furthermore, vasopressin can stimulate ACTH release in animals bearing

hypothalamic lesions that normally block stress responses (McCann and Fruit, 1957). Notably, although the nature of CRF is presently not known, it is possible to separate hypothalamic CRF from vasopressin during extraction procedures (Shally *et al.*, 1960; Dhariwal *et al.*, 1966).

The precise role played by vasopressin in regulating ACTH secretion in mammals is not known. Present hypotheses suggest that vasopressin acts to release ACTH directly and potentiates the action of hypothalamic CRF in stimulating ACTH release. Evidence in support of this hypothesis comes from several sources. In rats with a genetic defect which prevents the synthesis of vasopressin, an impairment in ACTH release is observed in response to submaximal stressors (Yates *et al.*, 1971). Of particular interest to the present study is the finding that lesions which cause diabetes insipidus in rats also suppress stress responses (McCann and Brobeck, 1954). Vasopressin given intravenously in rats in doses subthreshold for ACTH release greatly enhanced the effectiveness of a crude hypothalamic CRF preparation given intravenously. However, the latter did not potentiate the former in an experiment with a reversed sequence of injection (Yates *et al.*, 1971). In the same study, the potency of the hypothalamic CRF preparation was greatly enhanced in dehydrated normal rats. In addition, vasopressin has been reported to stimulate the release of endogenous CRF when placed in the medial basal hypothalamus (Hedge *et al.*, 1966).

The present work strongly implicates the neurohypophyseal peptides in the control of ACTH secretion in goldfish, and perhaps teleosts in general. The precise role of these neurohypophyseal hormones in

regulating ACTH secretion in the goldfish cannot be determined from this investigation. However, if they act in a manner similar to that established in mammals, they may act to cause the release of ACTH directly, the release of hypothalamic CRF, or to potentiate the action of hypothalamic CRF. Further studies are required to determine which of these hypotheses may best explain the role of neurohypophyseal hormones in regulating ACTH secretion in the goldfish.

Lesions in the epithalamus of goldfish which destroyed the habenular nuclei resulted in an enhanced stress response. This result indicates that the epithalamus may play some inhibitory role in controlling ACTH secretion in the goldfish. This inhibition may have been achieved in two ways. Firstly, the epithalamus may be the source of an inhibitory factor which may act to suppress ACTH secretion. An ACTH-adrenal inhibitor has been shown to exist in extracts of the cerebral cortex of the calf (Motta *et al.*, 1968). Hawkins and Ball (1973) reported that lyophilized acid extracts of rat cerebral cortex tissue significantly depressed the stress response of mollies bearing pituitary transplants. This suggests that the teleost pituitary-interrenal axis may be sensitive to inhibitory factors. However, neither the nature nor the mechanism of action of the inhibitory factor in the cerebral cortex of the calf or the rat has been identified, and there is no supporting evidence for such a factor beyond these observations.

A second interpretation of the results of lesioning the epithalamus is that these lesions may have interrupted neural pathways which may normally inhibit ACTH secretion by suppressing the release of CRF. In the rat, neural pathways that inhibit CRF release arise in the hippocampus and amygdala. In a recent review of the literature summarizing

neural pathways which play a role in the regulation of ACTH secretion Yates and Maran (1974) are forced to concede that at present, knowledge of the structure of the brain and particularly the limbic system is too incomplete to draw firm conclusions about the manner in which the hippocampus and the amygdala influence ACTH secretion. However, pharmacological research into the nature of these inhibitory pathways indicates that they are aminergic. Recent reviews of the literature relating brain catecholamines and ACTH secretion (van Loon, 1973; Yates and Maran, 1974) suggest that there is an adrenergic system which inhibits ACTH secretion, probably by inhibiting the secretion of CRF. In the goldfish brain, fiber tracts originating in the habenular nuclei descend to the thalamus (Schnitzlein, 1962). However, whether axons from these tracts extend to the hypothalamus of the goldfish and influence the activity of the cell bodies in the NLT and are related to the control of CRF secretion is presently not known. In addition, nervous pathways originating in the habenular nuclei ascend into the telencephalon (Schnitzlein, 1962). Whether these axons innervate the cells of the NPO and play a role in the control of the NPO-neurohypophyseal system is also not known.

In most vertebrates, the hypophysiotrophic hormones produced by the hypothalamus appear to be secreted into portal vessels which supply the pituitary gland. In teleost fishes, however, the axons of the hypophysiotrophic nerves pass directly into the anterior pituitary. Neurohormones released from these nerve fibres may reach the cells of the pituitary directly or may be distributed throughout the pituitary via intrapituitary blood vessels or basement membrane-lined intervascular channels (Bern *et al.*, 1971; Zambrano, 1972; Zambrano *et al.*, 1972;

Hawkins and Ball, 1973). Studies of the nature of the neurosecretory fibres innervating the goldfish pars distalis have demonstrated a dual neurosecretory innervation by Type A (Knowles, 1965) and Type B (Knowles, 1965) fibres. The Type A fibres are considered to arise in the preoptic nucleus. The Type B fibres are considered to originate, at least in part, from the cell bodies present in the NLT.

In the goldfish the ACTH cells have been shown to be innervated by Type B fibres (Leatherland, 1972; Kaul and Vollrath, 1974). In the present investigation, lesions of the NLT resulted in an impairment of ACTH secretion in the goldfish. This suggests that the NLT is the source of the Type B fibres innervating the ACTH cells. Zambrano *et al.* (1973/74) have proposed that the ACTH cells in *Tilapia mosambica* are under the control of Type A fibres. In the present investigation, lesions of the NPO (the source of the Type A fibre) resulted in an impairment of ACTH secretion in the goldfish. A role of the Type A fibres in regulating ACTH secretion in the goldfish may be expressed by the termination of these fibres in the vicinity of blood capillaries in the pituitary (Kaul and Vollrath, 1974). Another suggestion is that they may release their products into capillaries located in the choroid anterior to the pituitary stalk where they may be transported to the pituitary by portal vessels in the anterior pituitary stalk. Such a portal system has been described in goldfish by Peter (1973).

During the course of the lesioning experiments of the present investigation, several goldfish were lesioned in the pituitary stalk. Goldfish in which the pituitary stalk was completely sectioned were capable of responding to stress with an increase in serum corticosteroid

concentration. However, portal vessels supplying the pituitary were quite evident. This suggests that the release of ACTH from the pituitary in such fish may have been caused by the transport of CRF(s) from the hypothalamus to the pituitary via the portal vessels.

In the present investigation, lesions of the NLT of goldfish suppressed the increase in serum corticosteroids in response to three stressors--a sham-injection stress, a shallow-water stress, and a thermal stress. These stressors would be quite different in terms of the neural structures activated and it is assumed that different afferent pathways would be involved in bringing information concerning these stressors to the hypothalamus. In addition, the possibility exists that these stressors may have caused the release into the circulatory system of agents which may have acted to cause the release of CRF. For example, in mammals it has been shown that such blood-borne agents as vasopressin (Hedge *et al.*, 1966) and angiotensin (Gann, 1969) act on the hypothalamus to provoke the release of CRF. Due to the disparate means of input of the stressors used, the possibility that these lesions may have only interrupted afferent pathways to the cells which secrete CRF is highly unlikely, as lesions placed dorsal to the NLT and posterior to the NLT were ineffective in suppressing the stress response. The results of the NLT-lesioning experiments suggest that the NLT is the source of CRF in the goldfish hypothalamus because lesions around this area were ineffective. This is further supported by the histological observations that the ACTH cells in the goldfish are innervated by Type B fibres (Leatherland, 1972; Kaul and Vollrath, 1974) which arise, in part, from the NLT.

Lesions in the preoptic area which destroyed the NPO diminished

the stress response of goldfish subjected to a thermal stress or a sham-injection stress. The preoptic region, in mammals, has long been known to mediate thermoregulatory responses. The demonstration that lesions of the NPO diminished the stress response to both of these stressors indicates that the diminished stress response of the NPO-lesioned goldfish in response to the thermal stress was probably not due solely to the destruction of a preoptic area responsible for mediating the response to the thermal stress. Unfortunately, with regard to all three of the stressors employed in this work, information concerning neural pathways in the teleost brain which may be involved in the stimulation of CRF from the hypothalamus or the activation of the NPO is presently not available.

III. HORMONE PELLET IMPLANT EXPERIMENTS

The amount of hormone introduced into the brain is of critical importance to the experimental design of studies which attempt to localize areas of the brain responsive to the hormone. If the dose of hormone is excessive the results may be misleading due to a diffusion of the hormone away from the implant site to some other area which may be sensitive to the hormone. In the present investigations, hormone pellets containing approximately 0.3, 0.5, and 1.0 µg cortisol were implanted into the optic tectum--an area of the brain highly unlikely to be a negative feedback site for corticosteroids. Pellets containing 1.0 µg cortisol were considered to contain an excessive amount of cortisol because they suppressed the increase in serum corticosteroids of goldfish subjected to a sham-injection stress. Pellets containing 0.3 or 0.5 µg

cortisol implanted into the optic tectum did not suppress the stress response of goldfish. Pellets containing 0.3 or 0.5 µg cortisol were thus considered appropriate to investigate possible feedback effects of corticosteroids on various areas of the goldfish brain.

The results of the cortisol pellet implant experiments provide direct evidence for negative feedback effects of corticosteroids on the goldfish brain. Pellets containing 0.3 µg cortisol implanted into the NLT or preoptic recess of the third ventricle significantly suppressed the increase in serum corticosteroids normally observed in response to a sham-injection stress. Pellets containing 0.5 µg cortisol implanted into the pituitary gland, posterior hypothalamus, dorsomedial hypothalamus or lateral telencephalon rostral to the anterior commissure had no effect on the stress response of goldfish. However, pellet implants containing 0.5 µg cortisol in the NLT, preoptic recess of the third ventricle, or lateral telencephalon posterior to the anterior commissure suppressed the stress response of goldfish. These results indicate that the NLT and the preoptic-posterior telencephalon region are sensitive to feedback effects of corticosteroids in the goldfish.

Negative feedback effects of corticosteroids in inhibiting ACTH secretion in teleosts have been demonstrated in both *in vivo* and *in vitro* studies. As outlined in the Introduction, the results of *in vivo* studies conducted to date with various teleosts treated with metopirone, exogenous adrenocorticosteroids, mammalian ACTH, and the synthetic corticosteroids, betamethasone and dexamethasone, have all indicated that corticosteroids exert negative feedback effects to suppress ACTH secretion. However, in each of these approaches the site of action of the corticosteroid inhibition cannot be ascertained. Feedback inhibition may have occurred at the level of the hypothalamus, the pituitary gland, or

both. The results of the present study have clearly demonstrated that feedback effects occur in the NLT region of the hypothalamus and the preoptic-posterior telencephalon area.

Sage (1968) observed histologically an inhibition of corticotroph activity with cultured red swordtail pituitaries when corticosteroids were added to the culture medium. Dexamethasone and cortisol were the most effective inhibitors; however, only cortisol was effective at physiological concentrations (0.05-0.4 µg/ml). Similarly, Sage and Purrott (1969) reported a decrease in the spontaneous release of ACTH from cultured goldfish pituitary glands when cortisol was added to the incubation medium. However, this observation may be of pharmacological interest only as the concentration of cortisol in the incubation medium (5 µg/ml) was considerably greater than that observed under physiological conditions. The results of the investigations of Sage (1968) and Sage and Purrott (1969) demonstrate that corticosteroids may inhibit ACTH secretion *in vitro*. The results of the present investigations indicate that the site of negative feedback of cortisol in suppressing ACTH secretion in the goldfish *in vivo* is at the level of the brain and not the pituitary.

Feedback effects of corticosteroids in controlling ACTH secretion have been an area of intensive research in mammals. Corticosteroids have been shown to inhibit ACTH secretion when implanted in the medio-basal hypothalamus and pituitary gland (for a review see Yates and Maran, 1974). In the hypothalamus, corticosteroids presumably act to inhibit the synthesis and/or release of CRF. Takabe *et al.* (1972) have shown that the increase in the content of hypothalamic CRF that normally

occurs after stress in the rat can be diminished or abolished by the administration of dexamethasone prior to the application of the stressor.

At the pituitary, corticosteroids may act to inhibit the synthesis and/or release of ACTH. Dexamethasone, implanted into the pituitary, has been shown to decrease the amount of ACTH in the pituitary (Chowers *et al.*, 1967). However, in an *in vitro* study Fleischer and Rawls (1970) found that dexamethasone inhibited ACTH release but not ACTH synthesis. *In vitro*, corticosteroids have been shown to inhibit the release of ACTH normally observed in response to both vasopressin and hypothalamic CRF preparations (Fleischer and Vale, 1968; Kraicer *et al.*, 1970; Pollock, 1966). *In vivo*, corticosteroids given systemically diminished the potency of vasopressin and hypothalamic CRF preparations in intact animals (Russell *et al.*, 1969) and in rats with median eminence lesions (de Wied, 1964). Although corticosteroids have been shown to exhibit feedback effects on both the brain and the pituitary in mammals it is not known which site is the more important for corticosteroid inhibition under physiological conditions.

In the present investigations, cortisol pellet implants in the ventrobasal hypothalamus decreased the stress response of goldfish. These pellets were located in the NLTa and rostral NLTp. This is the same area which when lesioned also blocked the stress response. This suggests that in the goldfish corticosteroids may act on the hypothalamus to cause inhibition of the synthesis and/or release of CRF.

Cortisol pellet implants in the preoptic-posterior telencephalon region of the goldfish were also effective in suppressing the stress response. The results suggest that cortisol may influence either: 1) the activity of the NPO-neurohypophyseal system, which the lesioning

experiments have clearly demonstrated to play a stimulatory role in ACTH secretion, or 2) corticosteroid-sensitive neurons in this region which may in turn influence the activity of the neurosecretory cells of the hypothalamus which secrete CRF. With regard to the first interpretation of these results, it is interesting to note that an inverse relationship between the adrenocortical hormones and vasopressin secretion has long been recognized in mammals. Such observations are typified by the work of Ahmed *et al.* (1967) who demonstrated that the plasma vasopressin concentration is elevated following adrenalectomy and that it may be returned to normal by the administration of corticosteroids. Of particular interest to the present investigations are the observations of McCann *et al.* (1958) who found that the administration of corticosteroids diminished the antidiuretic response to stress and resulted in a decrease in the ADH (antidiuretic hormone) titer in the jugular venous blood of rats subjected to stress. These authors concluded that adrenal steroids suppress the release of vasopressin. Concerning the second interpretation of the present findings, several neural pathways in the goldfish brain descend from the telencephalon to the diencephalon. Two prominent pathways, the medial and lateral forebrain bundles, traverse the diencephalon (Schnitzlein, 1962). Axons from corticosteroid-sensitive neurons in the preoptic-posterior telencephalon region may descend via these pathways to the hypothalamus to influence the activity of the neurosecretory cells which secrete CRF. Further work is required to determine the manner in which cortisol can suppress stress responses by action on the preoptic-posterior telencephalon region.

Negative feedback effects of corticosteroids acting at the level of the hypothalamus have also been demonstrated in amphibians, birds

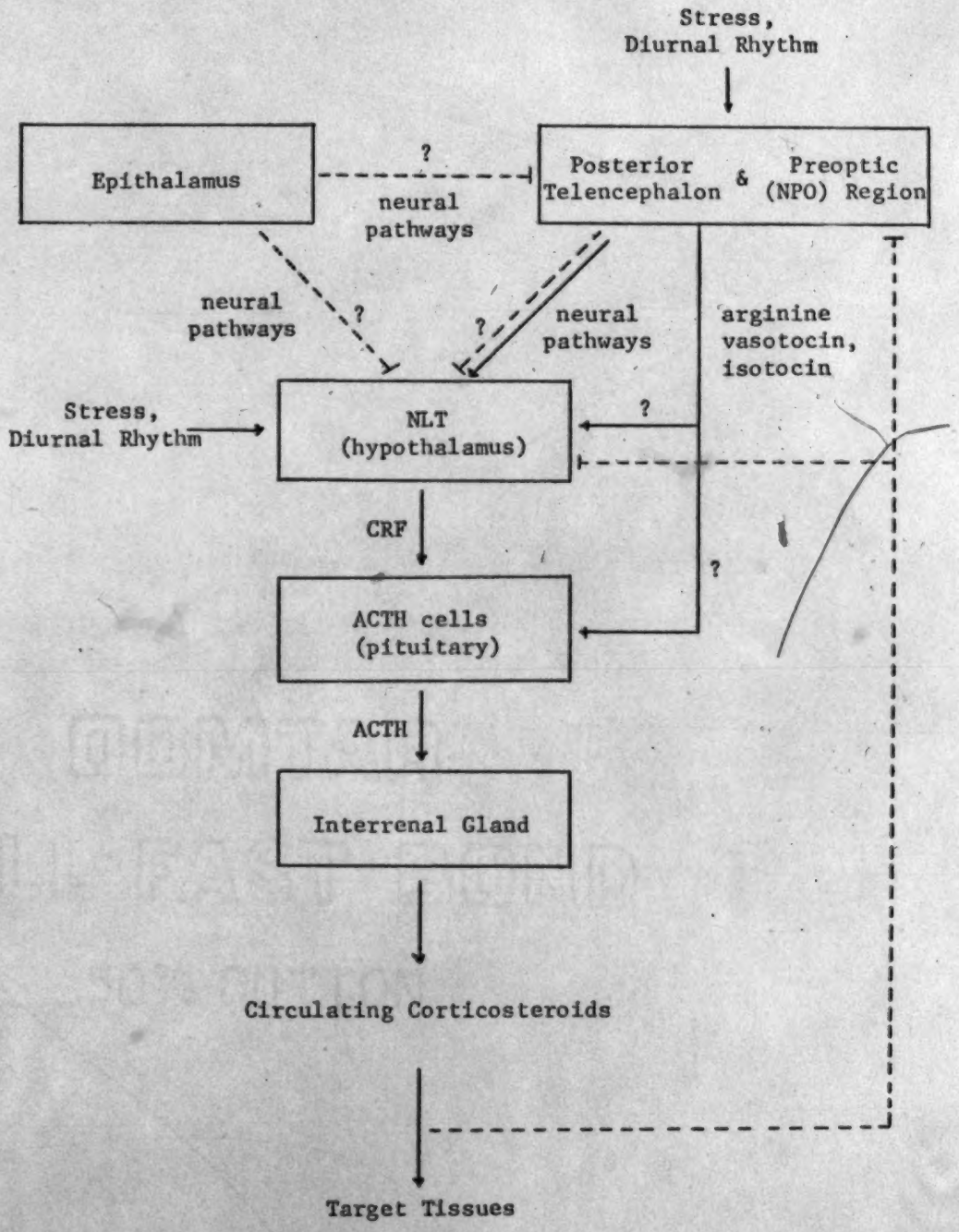
and reptiles. Laub *et al.* (1975) reported that both corticosterone and aldosterone implants in the anterior medial basal hypothalamus suppressed stress-induced increases in corticosteroid secretion in the anuran, *Rana pipiens*. In domestic fowl, Frankel *et al.* (1967) observed diminished stress responses when corticosterone was implanted in the hypothalamus in the vicinity of the infundibular nucleus. Callard *et al.* (1973) reported that in the lizard *Sceloporus cyanogenys*, betamethasone implants in the hypothalamus suppressed the adrenal hypertrophy observed following injections of metyrapone. In additional experiments, aldosterone and corticosterone implants in the anterior basal hypothalamic or median eminence reduced plasma corticosterone levels in another lizard, *Dipsosaurus dorsalis* (Callard *et al.*, 1973).

In the present investigation, cortisol pellet implants in the anterior mediobasal hypothalamus or the preoptic-posterior telencephalon region suppressed stress responses of goldfish. These results indicate that in all of the vertebrate classes, corticosteroid hormones exhibit feedback effects on the ventrobasal hypothalamus to inhibit ACTH secretion.

IV. CONCLUSIONS

The results of the present investigations make it possible to construct a model describing the control of ACTH secretion in the goldfish (Fig. 51). The hypothalamus is the source of a releasing factor (CRF) which can stimulate ACTH secretion from the pituitary both *in vitro* (Sage and Purrott, 1969) and *in vivo* (present investigation). Lesioning studies of the hypothalamus suggest that the source of CRF is the NLT.

Figure 51. A model describing the control of ACTH secretion in the goldfish. Solid lines indicate stimulatory relationships, broken lines indicate inhibitory relationships.


Stress,
Diurnal Rhythm
Epithalamus
?
neural
pathways
Posterior
Telencephalon
&
Preoptic
(NPO) Region
neural
pathways
?
?
neural
pathways
arginine
vasotocin,
isotocin
Stress,
Diurnal Rhythm
NLT
(hypothalamus)
?
CRF
?
ACTH cells
(pituitary)
ACTH
Interrenal Gland
Circulating Corticosteroids
Target Tissues

Lesions of this area of the hypothalamus suppress stress-induced ACTH secretion.

The NPO plays a stimulatory role in ACTH secretion as lesions of the NPO suppress stress responses. Additional evidence in support of an involvement of the NPO in regulating ACTH secretion of goldfish comes from the observation that extracts of the telencephalon, which included the NPO, of the goldfish and sucker stimulate ACTH secretion in the goldfish *in vivo*. The neurohypophyseal peptide hormones produced by the NPO, vasotocin and isotocin, may act to release ACTH directly from the pituitary, promote the release of hypothalamic CRF, or potentiate the action of CRF in promoting ACTH release.

The epithalamus plays an inhibitory role in the control of ACTH secretion. Lesions of the epithalamus which destroyed the habenular nuclei enhanced the response to stress. This result suggests that the epithalamus is the source of either a neurohormonal factor that inhibits ACTH release, or neural pathways which may normally inhibit ACTH secretion in some way. These inhibitory pathways may suppress the release of CRF from the NLT or suppress the activity of the NPO.

ACTH stimulates the synthesis and secretion of corticosteroid hormones from the interrenal gland. Corticosteroid hormones exhibit feedback effects on the brain to suppress ACTH secretion. Cortisol pellet implants in the hypothalamus or preoptic-telencephalon region suppressed the stress response of goldfish. This provides direct evidence for negative feedback effects of corticosteroids on the brain. The area of the hypothalamus responsive to feedback effects of corticosteroids appears to be restricted to the NLT as only pellet implants in this

region of the hypothalamus were effective. Cortisol pellet implants placed in the posterior telencephalon or preoptic (NPO) region suppressed the stress response of goldfish. This effect may be due to a direct action of cortisol on the cells of the NPO, or may be mediated by neural pathways descending to the hypothalamus to influence the activity of the neurosecretory cells which synthesize and release CRF. Cortisol implants in the pituitary were ineffective in suppressing ACTH secretion in a dose which was effective when implanted in the brain. This result indicates that the site of negative feedback of cortisol in suppressing ACTH secretion in the goldfish *in vivo* occurs not at the pituitary but at the brain, specifically in the NLT and preoptic-posterior telencephalon region. In addition, this feedback system may be overridden in response to various stimuli (stress, daily rhythm) which may act via the hypothalamus and/or NPO to promote further increases in ACTH secretion.

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APPENDIX

1012

Stress and adrenocorticosteroid dynamics in the goldfish, *Carassius auratus*

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Received January 6, 1975

FRYER, J. N. 1975. Stress and adrenocorticosteroid dynamics in the goldfish, *Carassius auratus*. Can. J. Zool. 53: 1012–1020.

In goldfish serum, cortisol was found to constitute 77.6% of the adrenocorticosteroids measured by a competitive protein-binding radioassay. Adult goldfish maintained on a photoperiod of 14 h light : 10 h dark in November exhibited no significant variation in serum corticosteroid concentration throughout the 24-h cycle. Goldfish maintained in an 8L:16D photoperiod in June exhibited two peaks in serum adrenocorticosteroid concentration. Four hours before the onset of the light period and 4 h after the onset of the light period, serum corticosteroids were significantly higher than those observed at the midpoint of the dark period. After sham injection, swimming in shallow water, or a thermal shock, but not a handling disturbance, circulating levels of corticosteroids were significantly higher than in undisturbed fish. Betamethasone injected 24 h before a thermal stress completely blocked the stress-induced increase in serum corticosteroids observed in vehicle-injected and uninjected goldfish, demonstrating the potency of this steroid as a blocker of the pituitary–interrenal axis in this species.

FRYER, J. N. 1975. Stress and adrenocorticosteroid dynamics in the goldfish, *Carassius auratus*. Can. J. Zool. 53: 1012–1020.

Dans le sérum du poisson rouge, l'hydrocortisone constitue 77.6% des adrénocorticostéroïdes mesurés par dosage radioactif impliquant la méthode de compétition. Des poissons rouges adultes gardés dans des conditions de photopériode de 14 h de lumière pour 10 h d'obscurité, en novembre, ne subissent pas de changement significatif de la concentration des corticostéroïdes du sérum dans un cycle de 24 h. Par contre, chez les poissons rouges gardés à une photopériode de 8 h de lumière et 16 h d'obscurité, en juin, on enregistre deux sommets dans la concentration des adrénocorticostéroïdes du sérum. Les concentrations des corticostéroïdes du sérum sont significativement plus élevées 4 h avant et 4 h après le début de la période de lumière qu'au milieu de la période d'obscurité. Après une injection simulée, une période de nage en eau peu profonde ou un choc thermique, les niveaux des corticostéroïdes en circulation sont significativement plus élevés que chez les poissons-témoins; cependant, la simple manipulation des poissons ne produit pas cet effet. De la bétaméthasone injectée 24 h avant un stress thermique bloque complètement l'augmentation, provoquée par le stress, des corticostéroïdes du serum observée chez des poissons rouges ayant subi une injection d'excipient ou n'ayant pas subi d'injection; ce phénomène démontre la propriété qu'a ce stéroïde de bloquer l'axe pituitaire–interrénal chez cette espèce.

[Traduit par le journal]

Introduction

Despite the wide use of goldfish in laboratory investigations, few studies have been reported describing stress-induced elevations in circulating adrenocorticosteroids in this species. Recently, Spieler (1974) reported an elevation (67%) in plasma cortisol observed 10 to 22 min after initial net capture and restraint. However, he observed no significant elevation in adrenocorticosteroids during the time interval 30 s to 10 min. These results contrast markedly with those reported in abstract form by Singley and Chavin (1971, 1972). They observed increases of 375% and 600% in cortisol levels of goldfish sampled 30 s (1971) and 15 s (1972), respectively, after the addition of crystalline sodium chloride to the aquarium water.

This investigation was undertaken to determine the diurnal range of serum corticosteroid levels in goldfish subjected to different photoperiods in the laboratory and to examine the magnitude of the elevation in circulating adrenocorticosteroids induced by various "stressors."

Materials and Methods

Adult goldfish of both sexes (25–50 g) were obtained from a commercial supplier and held for a minimum of 2 weeks before experimentation in 67.5-litre aquaria (six fish per aquarium) at a temperature of 20 ± 1°C. Water in the aquaria was filtered through glass wool and charcoal, and changed when required. All fish were subjected to a photoperiod of 14 h light : 10 h dark with the exception of those fish kept on a 8L:16D photoperiod in the diurnal rhythm study. Fish were fed daily with a commercially prepared diet, 1 to 2 h after the onset of the light period.

The blood sampling procedure was as follows. After all of the fish in the aquarium were caught with a single pass of a large net, they were immediately transferred to an anaesthetic solution (tricaine methanesulfonate 0.1‰, Kent Laboratories Ltd.). When opercular movement had ceased, the fish were removed from the anaesthetic solution and a pair of fine scissors was used to make a "V"-shaped cut about 1 cm posterior to the head extending down through the spinal cord and underlying dorsal aorta. Blood, upwelling into the cut, was collected in a 1.0-ml tuberculin syringe, and placed on ice in 12 × 75 mm polypropylene tubes for 45 to 60 min. After centrifuging at 2500 rpm for 10 min the serum was drawn off and stored at −25°C. All six fish in an aquarium could be sampled within 6 min of net capture. No correlation between order of sampling and serum corticosteroid concentration was observed. All blood sampling and experimental procedures, except as outlined below, were performed between 1200 and 1400 hours (4 to 6 h after the onset of the light period) to minimize the effects of diurnal variations in blood corticosteroid concentration.

Serum corticosteroids were determined by a competitive protein-binding radioassay (Murphy 1967), utilizing reagents from the Schwartz/Mann radioassay kit for cortisol (Schwartz/Mann, Orangeburg, N.Y.). Serum samples (100 µl) were extracted in 2.5 ml of methylene chloride by vortexing for 5 s and shaking for 10 min on a wrist-action mechanical shaker in capped 12 × 75 mm polypropylene tubes. Duplicate determinations were performed with 0.5-ml portions of the extracts transferred to additional 12 × 75 mm tubes. After evaporation to dryness with jets of nitrogen gas, 800 µl of buffer (0.04 M sodium monophosphate, pH 7.4), 100 µl of corticosteroid-binding globulin solution (a lyophilized preparation of transcortin in human plasma), and 100 µl of a tritiated cortisol solution (1.5 µCi in 5.0 ml of buffer) were added to the extract tubes and duplicate tubes of blanks and cortisol standards (0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 ng per tube) were used to prepare a standard curve. After heating in a water bath at 45°C for 5 min, the tubes were transferred to an ice-water bath. All subsequent steps for the assay were performed in a coldroom at 0°C. After an equilibration period of 30 min, 0.5 ml of a dextran-coated charcoal suspension (0.13 g in 60.0 ml of buffer) was added to each tube to separate unbound steroids from the protein complex. The tubes were then vortexed for 3 s and returned to the ice-water bath for 10 min before centrifuging at 2500 rpm for 10 min. A 1.0-ml aliquot of the supernatant from each tube was added to 15.0 ml of Bray's solution (Bray 1960), counted for 10 min in a liquid scintillation counter (Nuclear Chicago, Mark I), and the counts corrected for quenching. The amount of corticosteroid in each sample (nanograms per tube) was determined from the standard curve (% radioactivity bound vs. log nanograms per tube) and multiplied by 5 to give the number of micrograms per 100 ml of serum.

Although cortisol is the predominant corticosteroid of the goldfish (Chavin and Singley 1972) and of teleosts in general (Chester Jones *et al.* 1969), procedures were undertaken to quantify the amount of cortisol measured by the competitive protein-binding (CPB) assay for goldfish serum. The efficiency of the methylene chloride extraction of cortisol from serum was determined by recovering labelled cortisol added to goldfish serum. Five-, 10-, 15-, and 20-µl volumes of tritiated cortisol (3 µCi/0.5 ml, in ethanol) were added to 1.0 ml of ethanol or 1.0 ml of serum. The total amount of radioactivity added to the serum (cpm/100 µl) was determined by placing 100-µl aliquots of the isotope from the 1.0-ml volume of ethanol in a liquid scintillation vial. Labelled serum samples were extracted as described above and 0.5 ml of the extract placed in a scintillation vial. The contents of the total count and extract vials were evaporated to dryness under jets of nitrogen gas before the addition of 15.0 ml of Bray's solution. The vials were counted for 10 min and the counts corrected for quenching. The counts recovered from the serum samples were then multiplied by 5 and expressed as a percentage of the total number of counts added to the serum. Table 1 is a summary of the data determining the efficiency of the methylene chloride extraction of tritiated cortisol from goldfish serum. All data are presented as the means ± SEM. The mean extraction efficiency was 95.5 ± 1.9% and was independent of the amount of labelled steroid added to the serum.

TABLE 1

Summary of data (mean ± SEM) determining the efficiency of methylene chloride extraction of tritiated cortisol from goldfish serum. Numbers in parentheses indicate the number of determinations performed

Cpm added to serum, per 100 µl	Cpm recovered from serum, per 100 µl	% recovery
2274 ± 27 (4)	2134 ± 25 (7)	93.9 ± 1.1
4469 ± 64 (4)	4355 ± 163 (4)	97.5 ± 3.6
4195 ± 180 (4)	3949 ± 87 (4)	94.1 ± 2.1
6823 ± 79 (4)	6169 ± 146 (7)	90.4 ± 2.4
8990 ± 152 (4)	9132 ± 285 (7)	101.6 ± 1.9
Mean extraction efficiency		95.5 ± 1.9

Thin-layer chromatography (TLC) was used to separate cortisol from other steroids present in goldfish serum. Serum samples (100 µl) were extracted in 2.5 ml of methylene chloride and a 2.0-ml volume of the extract evaporated to dryness under jets of nitrogen gas. The residue was redissolved in 100 µl of 10% methanol in methylene chloride and spotted on 20 × 20 cm TLC sheets (No. 6060 Eastman silica gel chromatograms with fluorescent indicator) at points 2 cm apart along a baseline 2 cm from the bottom of the sheet. Cortisol markers (30 µg) were spotted at each end of the sheet. The sheets were then scored vertically, midway between each pair of spots, and placed in developing tanks containing 101 ml of the solvent chloroform–methanol–water (90:10:1) (Bennett and Heftmann 1962). After the solvent front had advanced 12.0 cm, the sheets were removed from the tanks and the cortisol markers visualized under ultraviolet light, with the sample lanes shielded from the light source. For each sample a 2 × 2 cm square of silica gel was aspirated from the sheet (Matthews *et al.* 1962) and eluted with 10 ml of 10% methanol in methylene chloride. A 2.5-ml volume of the extract was transferred to a 12 × 75 mm polypropylene tube, evaporated to

dryness under a jet of nitrogen gas, and assayed for cortisol.

To determine the amount of cortisol lost with the TLC procedure, tritiated cortisol was added to 1.0 ml of serum and 100-µl samples chromatogrammed as described above. The total amount of radioactivity (cpm/100 µl) added to the serum was determined as described above for calculating the efficiency of the extraction of labelled cortisol from serum. After chromatography, a 2.5-ml portion of the eluate was placed in a liquid scintillation vial and evaporated to dryness under a jet of nitrogen gas. After the addition of 15.0 ml of Bray's solution, the vials were counted, the counts corrected for quenching, and the radioactivity recovered from the TLC sheet expressed as a percentage of the radioactivity spotted on the sheet. The amount of radioactivity spotted on the sheet was the total count (6382 ± 43 cpm/100 µl; $N = 5$) less the counts lost through the extraction of the steroid from the serum. Correcting for the extraction efficiency (95.5 ± 1.9%), the mean number of counts spotted on the sheet was 6093 ± 41 cpm per sample. The mean number of counts recovered after thin-layer chromatography was 4894 ± 164 cpm per sample ($N = 7$), giving a mean recovery of labelled cortisol of 80.3 ± 2.7%.

To determine the proportion of cortisol in the total corticosteroids measured by the CPB technique, determinations were performed on a pool of serum taken from fish that had elevated corticosteroid titers induced by a thermal stress. Goldfish acclimated at 20 ± 1°C were netted, placed in a water bath at 35°C for 10 min, and returned to their holding tank. Blood samples were taken 30 min after the return of the fish to their holding tank. Assay of this serum pool directly, without thin-layer chromatography to separate cortisol from other corticosteroids, gave a corticosteroid titer of 17.4 ± 0.5 µg/100 ml ($N = 7$). After chromatographic separation of cortisol, the value obtained was 10.9 ± 0.5 µg/100 ml ($N = 7$). Correcting this value for procedural losses of steroid during TLC (19.7 ± 2.9%) gave a cortisol titer of 13.5 ± 1.0 µg/100 ml. Cortisol thus accounts for 77.6 ± 10.2% of the corticosteroids measured by this CPB technique. This percentage is similar to that obtained by Chavin and Singley (1972).

The ability of goldfish to respond to various stressors (sham injection, shallow water, temperature shock, and a handling disturbance) was assessed as follows. Goldfish were netted, sham-injected (insertion of a 27-gauge hypodermic needle 1.3 cm into the peritoneal cavity), and sampled 15 min after the return to their holding tank. The shallow-water stress consisted of netting the fish and placing them in a bucket containing water 2 cm in depth. For a temperature shock, goldfish were netted and transferred immediately into warm water (35°C). In one experiment, fish were sampled after spending 15 min at this temperature. In another, groups of fish were sampled at various periods after the return to their holding tank at 20 ± 1°C after spending 10 min in the warm water (35°C). The handling disturbance consisted of netting the fish and holding them completely out of the water for 60 s. Blood samples were taken at periods after the return of the fish to their holding tank.

The synthetic corticosteroid betamethasone (9-fluoro-11β,17,21-trihydroxy-16β-methylpregna-1,4-diene-20-dione) has been widely used to induce a pharmacological blockade of stress-induced elevations in circulating levels of adrenocorticosteroids in higher vertebrates (Smelik 1969). To assess the effectiveness of this steroid in blocking a stress-induced increase in corticosteroids in the goldfish, fish were injected with betamethasone (Sigma, 1.0 mg/kg in sesame oil, injection volume 4.0 µl/g) or sesame oil alone, 24 h before a thermal stress. Uninjected goldfish and fish injected with betamethasone or sesame oil were transferred to warm water (35°C) for 10 min and sampled 15 min after the return to the holding tank at 20 ± 1°C.

The data were analyzed using a one-way analysis of variance followed by Duncan's multiple range test.

Results

The serum corticosteroid levels of goldfish kept on two different photoperiod regimes are illustrated in Fig. 1. Fish maintained on the 14L:10D photoperiod (November 1973) exhibited no significant variation in serum corticosteroids throughout the 24-h cycle. The highest corticosteroid concentration (2.6 ± 0.3 µg/100 ml) observed at 0400 hours was not significantly greater than the lowest concentration (0.9 ± 0.4 µg/100 ml) observed at 1200 hours.

Goldfish kept on the 8L:16D photoperiod (June 1973) exhibited two peaks in serum corticosteroid concentration. The greater peak (6.3 ± 0.9 µg/100 ml) occurred at 0400 hours and was significantly higher than all other values, with the exception of the lower peak (4.7 ± 0.5 µg/100 ml) observed at 1200 hours. This lower peak was significantly higher than the lowest corticosteroid concentration (1.8 ± 0.5 µg/100 ml) observed at 2400 hours. No other statistically significant variations were detected.

The mean corticosteroid concentration for all of the fish kept on the 8L:16D photoperiod in June (3.7 ± 0.3 µg/100 ml) was significantly greater ($P < 0.001$) than that observed for fish on the 14L:10D photoperiod in November (1.8 ± 0.3 µg/100 ml).

The increase in serum corticosteroids of goldfish in response to various stressors is illustrated in Fig. 2. Fifteen minutes after sham injection, or swimming in shallow water, corticosteroid levels were significantly higher (9.4 ± 1.1 and 10.3 ± 1.7 µg/100 ml respectively) than those of undisturbed fish (1.6 ± 0.2 µg/100 ml). Goldfish placed in water at 35°C for 15 min before sampling had a corticosteroid titer (4.1 ± 0.6 µg/100 ml) that was not significantly greater than that of undisturbed fish. In addition, this value was significantly lower ($P < 0.05$) than that observed

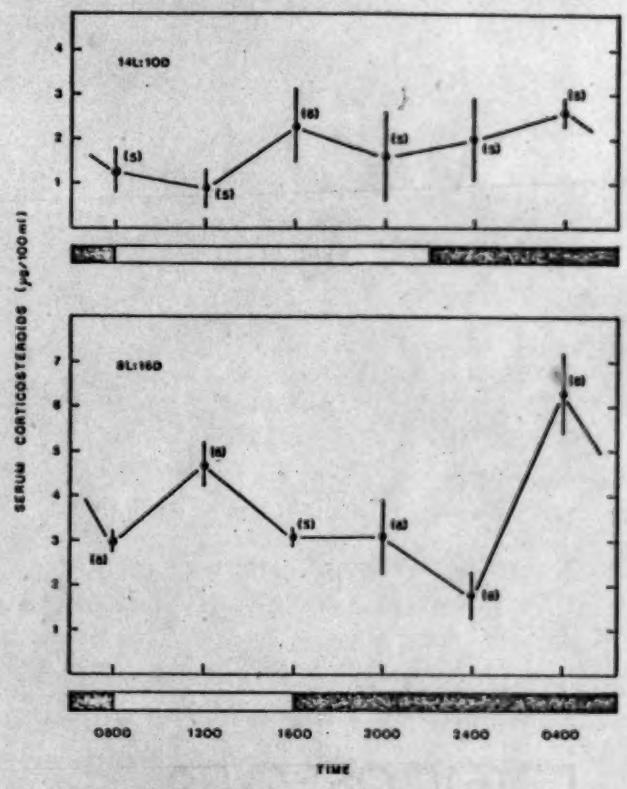

FIG. 1. Serum corticosteroid concentrations in goldfish kept on a 14-h or an 8-h photoperiod. Numbers in parentheses indicate the number of fish sampled. Vertical bars denote ±SEM.

with both the sham-injected fish and fish placed in shallow water. However, goldfish placed in water at 35°C for 10 min and then sampled 15 min after the return to the holding tank at 20 ± 1°C had corticosteroid levels (18.3 ± 3.5 µg/100 ml) that were significantly greater than those of undisturbed fish and of fish following the stress of sham injection or shallow water ($P < 0.01$).

The temporal relationship for corticosteroid levels of goldfish in response to a thermal stress and to a handling disturbance is summarized in Fig. 3. Serum corticosteroids of goldfish sampled 15 min after the return to their holding tank at 20 ± 1°C after a 10-min exposure to water at 35°C were significantly greater (13.7 ± 2.7 µg/100 ml) than those of undisturbed fish (1.9 ± 1.0 µg/100 ml). Serum corticosteroid levels of stressed fish were even higher at 30 min (16.3 ± 2.4 µg/100 ml), but by 60 min they had decreased to a value (8.0 ± 1.3 µg/100 ml) that was not significantly greater than that for undisturbed fish. In this experiment, the serum corticosteroid concentration at 15 min was lower, but not significantly lower, than that observed on two other occasions (Figs. 2, 4).

After a handling disturbance, serum corticosteroid levels increased only slightly to a maximum value (9.3 ± 1.6 µg/100 ml) observed 15 min after the return of the fish to their holding tank. This transient elevation was not significantly different from the corticosteroid levels obtained from undisturbed fish (7.0 ± 1.9 µg/100 ml). Corticosteroid titers were decreased at 30 min after the handling stress (7.2 ± 1.6 µg/100 ml) and remained higher than those observed previously (Figs. 2, 3). An explanation for this slight elevation is not known.

The effect of the synthetic steroid betamethasone on the increase in serum corticosteroid levels in goldfish after a thermal stress is illustrated in Fig. 4. The betamethasone-injected

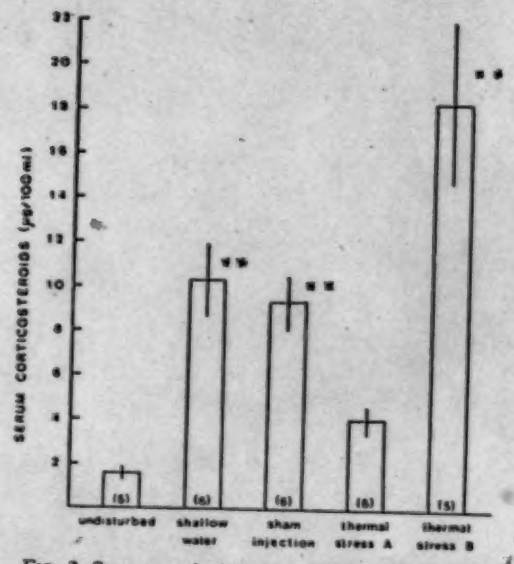

FIG. 2. Serum corticosteroid concentrations in undisturbed goldfish and goldfish sampled 15 min after subjection to shallow water, sham injection, water at 35°C (thermal stress A), and water at 35°C for 10 min followed by a return to the holding tank (thermal stress B). Numbers in parentheses indicate the number of fish sampled. Vertical bars denote ±SEM. Significant differences from undisturbed fish denoted by **, $P < 0.01$.

fish had corticosteroid titers (1.2 ± 0.4 µg/100 ml) that were significantly lower than those of both the uninjected fish (18.1 ± 2.4 µg/100 ml) and the sesame-oil-injected fish (18.9 ± 2.2 µg/100 ml). The sesame oil injection had no apparent effect on the magnitude of the increase in corticosteroids induced by the thermal stress. These results indicate that the injection of betamethasone before the thermal stress completely blocked the increase in serum corticosteroids normally induced by the stress.

Discussion

A CPB assay (Murphy 1967) has been used by several investigators to measure blood levels of corticosteroids in teleost fish (Bradshaw and Fontaine-Bertrand 1968; Fagerlund 1970; Ball *et al.* 1971; Srivastava and Meier 1972; Spieler 1974; Hargreaves and Porthé-Nibelle 1974). Cortisol is the predominant circulating adrenocorticosteroid of the goldfish (Chavin and Singley 1972) and of teleosts in general (Chester Jones *et al.* 1969). In this investigation, cortisol was found to constitute 77.6 ± 10.2% of the corticosteroids measured with the CPB assay. Chavin and Singley (1972) report the concentration of adrenocorticosteroids in a pool of goldfish serum as 44.0 µg cortisol, 7.2 µg corticosterone, 4.3 µg cortisone, 0.8 µg 11-deoxycorticosterone, and 0.11 µg aldosterone per 100 ml. This gives a percentage of 78.0 cortisol, a value very similar to the present results.

The CPB assay measures other corticosteroids to some degree. Comparing corticosteroid levels in goldfish plasma measured with the CPB assay with a double-isotope derivative assay for cortisol, Hargreaves and Porthé-Nibelle (1974) found that the CPB technique yielded slightly higher values. In addition, a good correlation between these two methods was obtained over a wide range of plasma corticosteroid concentrations. Although the results of this investigation indicate that the CPB method may not provide absolute concentrations of cortisol in goldfish serum, it is quite satisfactory for determining relative concentrations of corticosteroids in physiological studies.

In the present investigation, goldfish kept on the 14L:10D photoperiod in November exhibited no significant variation in serum corticosteroid concentration throughout the 24-h cycle. Goldfish subjected to the 8L:16D photoperiod in June, however, exhibited two peaks in serum corticosteroid concentration. Four hours before the onset of the light period and 4 h after the onset of the light period, serum corticosteroids were significantly higher than those observed at the midpoint of the dark period.

A diurnal variation in corticosteroid levels for goldfish has been reported previously. With goldfish sampled hourly on a 12L:12D photoperiod, Singley and Chavin (1975) observed two peaks in cortisol titer. The first peak was observed 3 to 4 h after the onset of the light period and the second 3 to 5 h later. In addition, two periods of low cortisol levels were observed. The first, of 2-h duration, separated the two cortisol peaks, and the second continued throughout the dark portion of the cycle and 2 h into the light period.

In the present study, the interval sample periods (4 h) were considerably longer than those used by Singley and Chavin (1975). The possibility remains that short-term fluctuations in corticosteroid concentration may not have been detected. Worthy of note, however, is the fact that a significant variation in corticosteroid levels was observed between various sampling periods with fish kept on the 8L:16D photo-

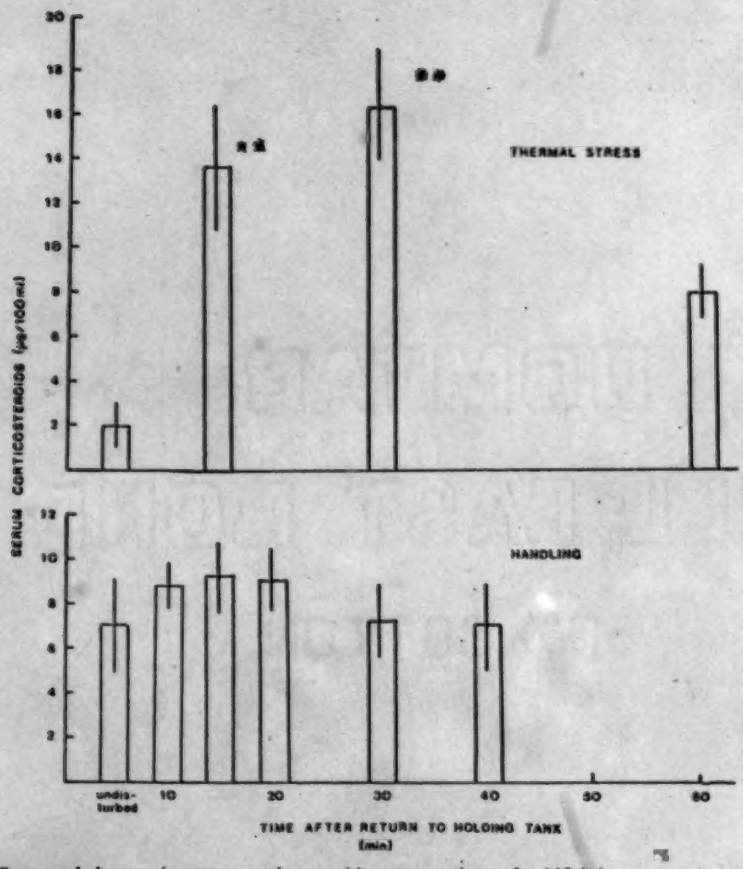

FIG. 3. Temporal changes in serum corticosteroid concentrations of goldfish in response to a thermal stress (netting and placing the fish in water at 35°C for 10 min) and to handling (netting and holding the fish out of the water in the net for 60 s) after the return of the fish to their holding tank. Each histogram represents the mean of five or six fish. Vertical bars denote ±SEM. Significant differences from undisturbed fish denoted by **, $P < 0.01$.

period in June, whereas no such variation was observed with fish kept on the 14L:10D photoperiod in November. In addition, the fish sampled in June maintained corticosteroid titers at a significantly higher level than those sampled in November. These differences may reflect a response to the length of the photoperiod or may be indicative of a seasonal difference in corticosteroid dynamics. Such possibilities require further investigation.

Daily rhythms of adrenocortical hormones occur widely in higher vertebrates. A literature summary describing the daily variations in the circulating levels of these hormones in various teleost fish is presented in Table 2. In these descriptions of diurnal variations, no pattern of similarity has been demonstrated. Evidence suggesting that the daily fluctuations in corticosteroids may be synchronized by the photoperiod has been reported by Srivastava and Meier (1972). They observed a peak in cortisol levels 8 h after the onset of the light period when the fish were kept on either a "normal" or an "inverted" 12-h photoperiod. Additional support for a photoperiodic control mechanism has been reported (unpublished observation cited by Redgate 1974). When the photoperiod was shifted by 6 h, peak cortisol levels were also displaced by this period of time. Before generalizations can be made for teleosts concerning diurnal variations in blood levels of corticosteroids, more detailed data must be obtained

TABLE 2

Summary of the literature describing daily variations in blood levels of corticosteroids for various teleosts indicating high and low corticosteroid concentrations. Unless indicated otherwise, numbers in parentheses denote the number of hours after the onset of the light period

Species	Photoperiod	High corticosteroid levels, µg/100 ml	Low corticosteroid levels, µg/100 ml	Author
Ictalurus punctatus	not stated, August	128 (2 p.m.)	68 (12 a.m.)	Boehlke *et al.* (1966)
Fundulus grandis	12L:12D, November	19.5 (8)	7.4 (4)	Srivastava and Meier (1972)
	12L:12D, (inverted) November	49.2 (8)	19.4 (4)	
Males only	natural, approximately	28 (1) and 27 (9)	19 (5) and 17 (17)	Garcia and Meier (1973)
Females only	0500 h to 1900 h, June and August	27 (1)	18 (9)	
Cyprinus carpio	12L:12D, March to May	25 (21)	0.3 (3)	Redgate (1974)
Carassius auratus	12L:12D	6.2* (4) and 4.4* (7–9)	1.3* (20)	Singley and Chavin (1975)
	8L:16D, June	4.7 (4) and 6.3 (20)	1.8 (16)	Present investigation
	14L:10D, November	2.6 (20)	0.9 (4)	

*Expressed as µg/100 ml per gram.

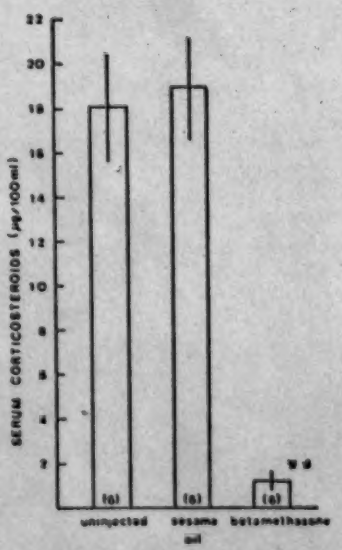

FIG. 4. Serum corticosteroid concentrations in non-injected goldfish and goldfish injected with betamethasone (1 mg/kg) in sesame oil, or sesame oil alone 24 h before a thermal stress. For the stress, fish were placed in water at 35 C for 10 min and sampled 15 min after the return to their holding tank. Numbers in parentheses indicate the number of fish sampled. Vertical bars denote ±SEM; **, denotes a significant difference from other treatments, $P < 0.01$.

throughout the year, both from fish in the laboratory held under a variety of photoperiods at several temperatures and from fish living under natural conditions in the wild.

Increases in the levels of circulating adrenocorticosteroids in teleost fish have been observed during spawning migrations (Idler *et al.* 1959; Hane and Robertson 1959; Schmidt and Idler 1962), after captivity of "wild" fish (Hane *et al.* 1966), exposure to metallic ions (Hill and Fromm 1968), surgery (Roy 1964), osmotic shock (Leloup-Hatey 1964; Hirano 1969), a change of habitat (Redgate 1974), forced swimming (Leloup-Hatey 1964; Hill and Fromm 1968), and during various disease conditions (Fagerlund 1967). These examples all reflect responses of fish to prolonged "stress" conditions. Relatively few reports describing increased corticosteroid levels as a result of an acute stress have been published. Short-term increases (within minutes) in corticosteroid levels have been reported after netting and restraint (Fagerlund 1967; Redgate 1974), lowering the water level in the holding tank (Wedemeyer 1969), a temperature shock (Wedemeyer 1969), and in response to an osmotic challenge (Singley and Chavin 1972).

In the present study, the increases in serum corticosteroids observed in response to sham

injection, shallow water, and a thermal stress are similar in magnitude to the increases in cortisol reported by Singley and Chavin (1971, 1972) for goldfish after the addition of crystalline sodium chloride to the holding tank. However, it is noteworthy to compare the corticosteroid levels of fish subjected to the different stress protocols. Goldfish sampled immediately after spending 15 min in water at 35°C had corticosteroid titers that were not significantly greater than those observed in undisturbed fish. In view of the magnitude of the elevation in corticosteroid levels induced by the 10-min exposure to water at 35°C followed by a return to the holding tank before sampling, this suggests that those fish kept at 35°C may have experienced a "thermal block" of the stress-induced increase in circulating corticosteroids. The site of such a block, whether at the level of the central nervous system, the pituitary gland, or the interrenal gland, requires further investigation.

Goldfish subjected to only a handling disturbance did not exhibit a significant increase in serum corticosteroids. A similar lack of a response was reported by Srivastava and Meier (1972) for the killifish, *Fundulus grandis*. Spieler (1974) found that a significant increase in corticosteroid levels occurred in goldfish only after metting and restraint for 10 to 22 min. Restraint for shorter periods of time had no effect on cortisol levels. It would appear that there is no significant change in the circulating levels of corticosteroids in goldfish after a brief handling disturbance.

The synthetic steroids dexamethasone and betamethasone have been used in several investigations of the pituitary–interrenal axis of teleosts. Donaldson and McBride (1967) observed that the injection of dexamethasone in rainbow trout, *Salmo gairdneri*, was followed by a reduction in plasma cortisol levels. Dexamethasone has also been shown to decrease corticosteroid levels in European eels (Bradshaw and Fontaine-Bertrand 1968) and in North American eels (Butler *et al.* 1969). Hawkins and Ball (1973) used betamethasone to block stress-induced increases in plasma corticosteroids of the molly, *Poecilia latipina*. In the present study, the failure of betamethasone-injected goldfish to respond to a thermal stress with increased corticosteroid levels demonstrates the potency of this steroid as a blocker of the pituitary–interrenal axis in this species.

In mammals, dexamethasone and betamethasone exert feedback effects at the hypothalamic level and at the level of the pituitary gland (Smelik 1969). However, in teleosts the feedback effects of corticosteroids have not been extensively investigated. Sage (1968) observed histologically an inhibition of corticotroph activity in cultured red swordtail (*Xiphophorus*) pituitaries when dexamethasone or cortisol was added to the culture medium. In cultured goldfish pituitaries, Sage and Purrott (1969) reported a decrease in release of adrenocorticotropic hormone in the presence of cortisol. The site of action of betamethasone in blocking the stress-induced elevation of corticosteroids in this study may have been at the level of the pituitary gland, the hypothalamus, or both. To date no information is available concerning possible feedback effects of adrenocorticosteroids on the hypothalamus of teleosts. Investigations are presently in progress to determine if these hormones exhibit feedback effects on the hypothalamus of the goldfish.

Acknowledgments

I am grateful to Dr. R. E. Peter for reviewing this manuscript and for many fruitful discussions throughout this investigation. This research was funded by grant A6371 from the National Research Council of Canada to Dr. R. E. Peter.

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